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Dated: October 31, 2007 Signature: _____ (Juan Quintero)

Docket No.: 29853/37702 (PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Shuyuan Zhang et al.

Application No.: 10/033,571 Confirmation No.: 97140

Filed: December 27, 2001 Art Unit: 1648

For: AN IMPROVED METHOD FOR THE PRODUCTION AND PURIFICATION OF ADENOVIRAL VECTORS Examiner: B. P. Blumel

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF PETER CLARKE, Ph.D. UNDER 37 CFR 1.132

I, Peter Clarke, PhD, hereby declare as follows:

1. I have been employed by Introgen Therapeutics Inc. for three years and my current position with this company is Vice President of Production and Technical Processes. Furthermore, I have over 25 years of experience in the biotechnology field working in both research and manufacturing. I have held positions at numerous biotechnology and pharmaceutical companies such as Director of Manufacturing at Chiron and Bayer Biological Products. In addition, my educational background includes a B.Sc. in biochemistry at Sheffield University in England, a Ph.D. in microbial physiology from the University of London, UK and a DIC in biochemistry from the Imperial College of Science and Technology in London, UK. A copy of my curriculum vitae is attached as Exhibit 1.

2. I understand that in reference to U.S. Patent Application Serial No. 10/033,571 (the '571 application). The Examiner has taken the position that a skilled person facing the technical problem of making pharmaceutically acceptable adenovirus composition would arrive at the process exemplified by claim 70 in view of the combined teachings of Shabram et al. (US Patent 5,837,520), Huyghe et al. (Human Gene Therapy, 1995), Kozak et al. (developments in Biological Standardization, 1996), Keay et al. (Biotechnology and

Bioengineering, 1976), Nadeau et al. (Biotechnology and Bioengineering, 1996) and Griffiths (Animal Cell Biotechnology, 1986).

3. From my understanding, the Examiner has taken the position that Nadeau teaches cell culture methods for "scale-up" of adenoviral preparations including methods employing "fed-batch" cell culture. Despite this assertion the Examiner apparently recognizes that Nadeau does not teach adenoviral production itself (see the March 16, 2007 Office Action at page 5). I have reviewed Nadeau and several other references discussed herein, and as a scientist in the field of biotechnology, I do not believe that a skilled person would apply the teachings of this reference to adenovirus production or purification. Although Nadeau at the beginning of both the abstract and the introduction refers to protein and virus production, the data shown in Nadeau are exclusively restricted to the production of recombinant proteins. My review of Nadeau, other journal articles, and my own experience in the field of biotechnology form the basis of my conclusion that the person skilled in the art would not have considered that the *protein production* methods of Nadeau to be equally applicable to *virus production* and certainly they would not have been considered applicable to methods of virus purification. Furthermore, even if it were believed that Nadeau was relevant to virus production, the teachings of Nadeau would lead the skilled artisan away from the teaching of claim 70 of the above-identified application, namely a method for making a *pharmaceutically acceptable* adenovirus composition comprising providing nutrients to host cells by perfusion or through a fed-batch process prior to infection.

4. In the next sections, I will discuss in detail why it is evident to me, as it would be to others skilled in the art of adenovirus production, that the data illustrated in Nadeau concern protein production in host cells and not adenovirus production by host cells. Furthermore, I will discuss why the protein expression methods taught by Nadeau are not applicable to methods for adenoviral production or purification.

5. First, Nadeau studies protein production in cells mediated by an unusual adenoviral expression system. The PTPIC transgene is expressed from a modified adenoviral major late promoter (MLP). This promoter type is also used by adenovirus for expressing the "late" viral proteins required for production of viral particles. Since Nadeau indicates that the PTPIC transgene is positioned in a recombinant expression cassette under the control of a recombinant MLP, (see Exhibit 2, Garnier *et al.* Cytotechnology, 15:145-

155, 1995, at pages 145-46) the adenoviral vector must also include a second MLP, the natural adenoviral MLP, in order to express genes encoding for the "late" viral proteins. Upon further review, I found that this particular vector is derived from an adenovirus construct in which the recombinant MLP (controlling expression of PTP1C) contains additional enhancer sequences. In fact, Massie *et al., Bio/Technology*,13:602-608, 1995 (Exhibit 3), from which the adenoviral vector of Nadeau is derived, states that "the human BKV enhancer, which has been shown to activate the Ad MLP in several cell lines and more efficiently in 293, was inserted at position -256 relative to the MLP transcription start site," (Massie *et al.* at page 603). This enhanced MLP is therefore different from the natural adenoviral MLP. First, the addition of enhancer sequences would add new factors pertaining to transcriptional regulation in comparison to the natural adenoviral MLP. Second, with such a vector construction, the level of viral proteins normally expressed from the natural adenoviral MLP (and consequently, also the level of adenovirus produced) would necessarily be unequal (and much lower) in relation to the level of protein expressed by the enhanced MLP. All of the assays to determine optimal cell culture conditions measure only the production of protein as mediated by the enhanced MLP. The authors of Nadeau never quantified viral production in their system, even though this would have been a straightforward and routine assay. Thus, there is no scientific basis in Nadeau for concluding that virus production correlates with protein production; on the contrary, it is much more likely that protein production as described in Nadeau does not correlate to virus production. Thus, the skilled artisan seeking a method for producing pharmaceutically acceptable adenovirus would not be motivated to look to Nadeau because Nadeau does not provide a teaching that is germane to adenoviral production.

6. There is also strong evidence that the recombinant protein production studied in Nadeau does not correlate with viral production. This is because the cells used for protein production in Nadeau show a significant metabolic activity, while in cells producing adenoviruses, such metabolic activity would not be expected by the person skilled in the art. First, Figure 5 of Nadeau shows that the host cells exhibit continued protein production well past 48 hours post-infection. Furthermore, Nadeau states that "lactate is quantitatively the major metabolic byproduct of the cells ..." (Nadeau, page 614, left column, third paragraph). Figures 6b, 7b and 8b indicate that after infection, the level of lactate in cell cultures continues to increase past 48 hours. Thus, Nadeau clearly teaches that the cells producing the

recombinant proteins exhibited a high degree of metabolic activity well past 48 hours post infection. However, it is well known that adenovirus-infected cells (or more particularly, cells in which adenoviruses are replicating) should show dramatic decreases in cellular viability and metabolic activity prior to 48 hours post infection. For example, Zhang and Schneider, *J Virol.*. 68:2544-2555, 1994 (Exhibit 4) indicates that "293 cells are exquisitely sensitive to late Ad CPE [adenovirus cytopathic effects]...and generally displayed severe morphological alterations such as swelling, detachment from the monolayer and lysis by 24 to 48 11 after infection." *(Id*,page 2456. 2nd paragraph). Taken together, it is my opinion that the evidence of cellular metabolism and protein production does not correlate with the normal indications of a "productive" adenovirus infection *(i.e.,* cell growth and infection that leads to adenovirus production as opposed to protein production). It is my understanding that the cells in Nadeau have been manipulated such that they produce high amounts of proteins (as described *supra*), but only little, if any, amounts of virus. Thus, cell growth conditions defined by Nadeau, while relevant to protein production would not be recognized as applicable to adenovirus production or purification.

7. I have also reviewed Cote *el al., Biotechnol. Prog.,* 13:709-714, 1997 (Exhibit 5), a subsequent paper which shares three authors in common with Nadeau. Excerpts of this paper were first presented at the Williamsburg Bioprocessing Conference about the same time the '677 priority application was filed, in November 1996. Using a different promoter, the Cote reference confirms that recombinant protein production does not typically correlate to viral production. The authors prepare and test an adenoviral vector comprising a green fluorescent protein (GFP) transgene positioned under the control of a cytomegalovirus (CMV) promoter. They report that similar levels of GFP were produced by this adenovirus at similar rates under all conditions. (See *e.g.,* page 712, right column, 2nd paragraph). Yet at the same time, static-flask cultured 293 cells produced 350 infectious virus particles/10^6 total cells, whereas shake flask cultured 293 cells produced only 220 infectious virus particles/10^6 total cells. Thus, while both cultures where in essence batch, one culture produced about 50% more infectious viral particles than the other culture even though transgene expression appeared the same. From this data one could conclude that recombinant protein production does *not* appear to correlate with production of infectious viral particles and thus the teaching of Nadeau is not applicable to adenoviral production.

8. Furthermore, one seeking to enhance or "scale-up" viral production would not be directed to Nadeau, which is directed to a technique that would promote enhanced cell growth. In this regard the attention of the reader is directed to Nadeau, and in particular the studies reported in Table I, which show that the technique giving the highest number of cells prior to infection actually gave the *lowest* amount of recombinant protein production per cell. The right most column of this table shows the data for the cells grown to the highest cell density prior to infection (3.0×10^6 cells/ml). However, this use of fed-batch culture techniques *prior to* infection resulted in the lowest specific protein production rate (only 26.6 $\mu g/10^6$ total cells). In contrast, with a batch technique prior to infection, nearly the same amount of proteins could be obtained with about half of the cells (see Table I, left column). Apparently, the cell number is not a major parameter for protein production according to Nadeau. Thus, while Nadeau teaches how to increase protein production it provides no teaching that would be applicable to "scale-up" of adenovirus production, because it is completely silent on virus production.

9. Since, as discussed above, Nadeau fails to provide teaching relevant to methods for adenoviral production it also completely fails to provide any teaching relevant to adenoviral purification. Thus, even if protein production described by Nadeau could be correlated with viral production, Nadeau provides no teaching regarding possible enhancements to adenoviral purity that may be achieved by altering growth conditions for host cells prior to infection with adenovirus.

10. In summary, based on my knowledge and experience and in light of the facts set forth above, I am firmly of the opinion that the skilled worker in 1996, upon a review of Nadeau et al., would conclude:

- That Nadeau is at most, only relevant to recombinant protein production, not adenoviral production.

- That recombinant protein production does not correlate with productive viral production.

- That Nadeau does not teach how to enhance adenovirus production by altering cell culture conditions.

- That Nadeau provides no teaching that is applicable to methods for purifying adenovirus.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of any U.S. patent issued in this application.

Date: 26 October 2007 By: _____

Peter Clarke, Ph.D.

Exhibit 1

Curriculum Vitae - Dr. Peter Clarke

AGE:	46 (DOB 15/12/59)
NATIONALITY:	British
MARITAL STATUS:	Married with three children
ADDRESS:	13911 Wilde Forest Court, Sugar Land, TX 77478 USA
Tel Home:	(713) 589 4824
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Educational background:
1985 Ph.D. in Microbial Physiology from London University.
1985 D.I.C. in Biochemistry from Imperial College of Science and Technology.
1981 B.Sc. (II,i) in Biochemistry from Sheffield University.

Language Skills:
Italian - After working in Italy for almost 5 years I have a good working knowledge of both written and spoken Italian.

CAREER SUMMARY

Present Employer: Introgen Therapeutics, Inc.
2250 Holcombe Boulevard,
Houston, TX 77030

Feb. '04 – Present: VP Production and Technical Processes

Responsible for, Manufacturing, Quality Control, Facilities and Engineering, Materials Management, Process Development and Process Sciences

May '00 – Feb. 2004: Director of Manufacturing — Bayer Biologicals
Raleigh, NC

Bayer Biologicals is, through Bayer Healthcare, part of Bayer Ag. It has manufacturing sites in California, North Carolina and a packaging facility in Rosia, Italy. Its main business is the manufacture and sale of therapeutic proteins derived from blood plasma.

I was initially recruited into the Bayer Biologicals facility in Rosia Italy. I worked there for 14 months, transferring packaging operations for all of Bayer Biologicals' products sold in Europe from Germany to the Rosia facility. This involved extensive travel within Europe and liaison with the manufacturing facilities in the US dealing with the regulatory and logistical aspects of the transfer and ensuring smooth ramp-up of operations in the new Italian packaging facility. By May 2001 all of Bayer Biologicals' products for sale in Europe were being packaged at Rosia.

During this period I was also the Operations representative on a project team designing a new 3000 m2 facility to be built in Rosia for the production of a sterile immunoglobulin derived from fractionated plasma. The project concluded at the end of detailed design when the Bayer board made the strategic decision to concentrate efforts in immunoglobulin purification at its new 4500 m2 facility in Clayton, North Carolina.

I transferred to the US in August 2001 and was responsible for taking the immunoglobulin purification facility through the final stages of validation, and aided in the production, submission and presentation of regulatory documents to the US, Canadian and European agencies. After licence submission I worked to prepare the facility and staff for pre-licence inspections by all the targeted Regulatory Agencies.

The product and facility were granted marketing licences in the USA and Canada in August 2003. The manufacturing department is now fully integrated into the Clayton site and has grown to a headcount of 125 people from 9 in January 2002. Routine production is currently underway to sustain product launches.

Sep. '98 - May '00:- Project/Product Manager

Medeva Pharma ltd.,
Liverpool,
UK

Medeva Pharma is the European Operations division of Medeva plc, a global pharmaceutical company. The Speke site employs approximately 700 people and produces a range of pharmaceuticals for sale in all regions of the world.

I worked as Project Manager for Hepacare, Medeva's novel, recombinant hepatitis B vaccine. In this role I was responsible for a number of projects involved in the first commercialisation of Hepacare and the construction, commissioning and qualification of a larger scale production facility. As these projects reached a successful conclusion, I became the Product Manager for Hepacare having sole responsibility for Hepacare manufacturing.

The commercialisation of Hepacare required that I liaised with Medeva Group Development and Medeva Pharma transferring the process, quality systems and existing facility from these two disparate organisations within Medeva plc. My objectives included the constitution of a functioning Project Team tasked to drive the validation of the production facilities, the compilation of the regulatory submissions in support of Hepacare and the recruitment of staff into Medeva Pharma in support of a successful product launch. This work resulted in the granting of a Marketing Authorisation for Hepacare from the EMEA.

In my second project (the construction and validation of a larger scale facility) I was responsible for a multi-million pound budget and co-ordinated the activities of a number of functions both within Medeva and with contractors. The facility was designed to comply with both European and US regulations governing aseptic and sterile processing and included the construction of both process and clean utility areas.

Mar. 98 - June 98 :- Product Development and Operations Director — SIFI SpA, Catania, Sicily, Italy

SIFI is a small Ophthalmic pharmaceutical company employing 300 staff and is the niche market leader within Italy. In my role at SIFI I was responsible for about 180 staff working in Manufacturing, Logistics, Quality, Regulatory Affairs and Clinical Studies.

Soon after starting with the Company I initiated the process of implementing production planning philosophies and expanding SIFI's products into other European markets. It was during this latter work that I discovered Company practices that I couldn't condone.

I worked with the Company's owners to produce action plans aimed at the elimination of these practices, but after four months with no commitment to change being made, I regretfully felt that I had to immediately resign my position with the Company.

Feb. 95 - Feb. 98 :- Director of Primary Production — Chiron SpA, Siena, Italy

Chiron SpA was founded in 1904 (as SCLAVO) with it's main business being the manufacture and sale of vaccines. The Company, an integral part of Chiron Vaccines, employs approximately 700 people and works within a heavily unionised environment.

I was initially recruited into Chiron SpA to head a Technical Support Group, but within 6 months had been promoted to Head of Manufacturing for Bacterial Bulk Vaccines (Siena) and eventually to Director of Primary Production, where I had responsibility for

the operation of 5 production plants producing both traditional and recombinant pharmaceuticals. The products from the manufacturing facilities under my control were distributed to a global market, but sales were concentrated mainly in Europe, the Americas and the Far East. Value of sales for these products was in the order of many tens of millions of dollars per annum.

My work within the Production Department consisted of developing the department's organisation and carrying out a series of Technical, Quality and Capacity audits from which we constructed and executed a series of improvements. As a result of action plans developed from these audits, quality systems were developed that brought the Company back into compliance and allowed us to successfully withstand an FDA inspection. I was also heavily involved in the launch of a novel, recombinant vaccine in Europe. Working with an international project team to very tight schedules I was responsible for activities carried out in the functional areas of Manufacturing, Quality and Engineering. The work included making presentations to regulatory agencies and contributing to documents to be submitted through the centralised application procedure of the EMEA, the production of clinical trial and product launch batches and organising the preparation for the audit of facilities. Prior to my departure from Chiron SpA the Danish and Italian regulatory agencies inspected the facilities, and shortly after, marketing authorisations were granted by the EMEA.

Aug. 1986 - Feb. 1995 :- Deputy Plant Manager — Delta Biotechnology
Nottingham
UK

My first position in Delta was as a Research Scientist where I was involved in the setting up of the Fermentation Development department. This involved the recruitment of a team of research scientists and technicians and equipping two fermentation laboratories with a range of chemostat and fed-batch fermenters, analytical equipment and development of a SCADA system. Thereafter, leading a team of research scientists and Technicians, my work included the definition of optimum process conditions for a range of organisms producing recombinant proteins. Predominately we worked on strains of Saccharomyces secreting the human blood protein HSA (rHA) , work for which we have been granted a patent. I was given the opportunity of continuing my association with the rHA project by moving to the company's production facility.

My final role at Delta was as deputy Plant Manager at Delta's production facility at Nottingham, which purified Human Serum Albumin derived from a genetically engineered yeast fermentation process. My role within the plant developed along with the facility, being involved in it's design, commissioning and operation. For the majority of this time I led a team of Process Workers, Technical Support and Warehousing staff and was responsible for the developing and successfully implementing production plans.

This objective having been completed I was involved in projects to increase the plant's capacity and to improve quality through a process of continuous improvement. Work

towards this goal involved the identification of alternative production strategies, the implementation of a new organisation and development of technologies that led to a 10 fold increase in productivity. My involvement in these activities was extensive and included the overhaul of the warehousing function, the generation of a comprehensive production documentation system, recruiting additional staff to operate a new 24 hour shift system and a 6 month period in Canada working with contractors to develop a CIM package and other software systems for the Plant.
The Plant had successfully completed its start-up and was in full production when I was offered a position with Chiron SpA in Siena.

Aug. 1984 - Aug. 1986:- Products Manager — ICI Biological Products, Billingham, UK

My first role with ICI Biological Products was as a Research Scientist leading a team of Technicians in the Fermentation department developing production processes for a number of potential products. I worked on a number of fermentation processes including Lactobacillus and Aspergillus cultures but concentrated mainly on a Fusarium fermentation which produced the cyanide degrading enzyme, cyanide hydratase ('Cyclear') and was granted a patent covering this work. My association with this product continued when I was promoted to Products Manager .

As Products Manager I was responsible for the identification and development of new products. I was most closely associated with the cyanide degrading product 'Cyclear' but worked on a number of other enzyme-based products. I was required to research markets, co-ordinate research and development activities and to arrange customer trials.

1981 - 1985 Ph.D. in Microbial Physiology from London University.

My thesis was based on the investigation of the physiological changes associated with the adaptation of E.coli to anaerobiosis. The work involved the construction of strains bearing specific lesions in oxidative and fermentative pathways of glucose metabolism by a combination of classical and molecular genetics and the biochemical analysis of their responses to varying degrees of anaerobiosis.

MEMBERSHIPS:

International Society of Pharmaceutical Engineers.
Chartered Biologist of the Institute of Biology

EDUCATIONAL BACKGROUND

Ph.D. Microbial Physiology
London University, UK (1985)

D.I.C Biochemistry
Imperial College of Science and Technology, London, UK (1985)

B.Sc. Biochemistry
Sheffield University, UK (1981)

CAREER SUMMARY

Feb '04 - Present Introgen Therapeutics, Inc. Houston, TX	**VP Production and Technical Processes**
May '00 – Feb '04 Bayer Biologicals, Clayton, NC & Siena, Italy	**Director of Manufacturing**
Sep. '98 - May '00 Medeva Pharma ltd., Liverpool, UK	**Projects Manager**
Feb. 95 - Feb. 98 Chiron SpA Siena Italy	**Director of Primary Production**
Aug. '86 - Feb. '95 Delta Biotechnology Nottingham UK	**Research Scientist to Deputy Plant Manager**
Aug. '84 - Aug. '86 ICI Biological Products Billingham UK	**Research Scientist**

Exhibit 2

Cytotechnology 15: 145–155, 1994.

Scale-up of the adenovirus expression system for the production of recombinant protein in human 293S cells

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Key words: Adenovirus, human 293S cells, recombinant protein, scale-up, metabolism

Abstract

Human 293S cells, a cell line adapted to suspension culture, were grown to 5×10^6 cells/mL in batch with calcium-free DMEM. These cells, infected with new constructions of adenovirus vectors, yielded as much as 10 to 20% recombinant protein with respect to the total cellular protein content. Until recently, high specific productivity of recombinant protein was limited to low cell density infected cultures of no more than 5×10^5 cells/mL. In this paper, we show with a model protein, Protein Tyrosine Phosphatase 1C, how high product yield can be maintained at high cell densities of 2×10^6 cells/mL by a medium replacement strategy. This allows the production of as much as 90 mg/L of active recombinant protein per culture volume. Analysis of key limiting/inhibiting medium components showed that glucose addition along with pH control can yield the same productivity as a medium replacement strategy at high cell density in calcium-free DMEM. Finally, the above results were reproduced in 3L bioreactor suspension culture thereby establishing the scalability of this expression system. The process we developed is used routinely with the same success for the production of various recombinant proteins and viruses.

Abbreviations: CFDMEM – calcium-free DMEM; CS – bovine calf serum; hpi – hours post-infection; J+ – enriched Joklik medium; MLP – major late promoter; MOI – multiplicity of infection (# of infectious viral particle/cell); q – specific consumption rate (mole/cell.h); pfu – plaque forming unit (# of infectious viral particle); Y – yield (μg/E6 cells or mole/cell)

Introduction

The technological potential of adenovirus vectors (AV) in various applications such as 1) recombinant protein production, 2) live viral sub-unit vaccines production and 3) gene transfer for establishing stable cell lines or for gene therapy (reviewed in Berkner, 1988, 1992; Gerard and Meidell, 1993; Graham and Prevec, 1992) currently gives rise to growing interest from biotechnologists. All of these applications will require the production of large quantities of either recombinant proteins or AV stocks. However, so far, no significant research efforts have been directed towards the scale-up of the AV expression system.

Helper-independent AVs were developed in the early 80's for high-level expression of recombinant proteins in human cells. By deleting the E1 and E3 regions of the adenovirus genome, transcription cassettes up to 7.0Kbp could be inserted in AV (Gluzman *et al.*, 1982). While the deletion of the E3 region only affects the ability of the AV to efficiently propagate in whole animals (Berkner, 1988), the deletion of the E1 region prevents it's replication in all mammalian cells (either in vivo or in vitro). However, AV lacking the E1 and E3 regions can be propagated in the human 293 cell line which constitutively expresses the adenovirus E1 polypeptides (Graham *et al.*, 1977, Berkner, 1988). Thus, the AV/293 expression system has a double lock security feature built-in that restricts the propagation of replication defective recombinant viruses to the complementing 293 cell line. Typically, the construction of replication defective AV for recombinant protein production is accomplished by inserting Major Late promoter-based (MLP) expression cassettes in place of

the deleted adenovirus E1 region. The adenovirus MLP is one of the strongest mammalian promoters and its transcriptional activity is responsible for the accumulation of the abundant adenovirus late proteins which represent collectively as much as 30–40% of total cellular proteins in adenovirus-infected cells (Ginsberg, 1984). However, due to the complexity in the regulation of gene expression in adenovirus, the recombinant protein production, using the first generation of expression vectors, has never exceeded 4% of the total proteins (estimated from data in Berkner, 1992). Consequently, the development of the full potential of AV as a high-level expression system has lagged behind other similar expression vectors such as the baculovirus/insect cells system (reviewed in O'Reilly *et al.*, 1992).

Recently, we have reported the construction of a new adenovirus expression vector (pAdBM5) that allows for the production of unprecedented levels of recombinant protein in AV-infected 293 cells (Massie *et al.*, 1994). In 293 cells infected with AV derived from the pAdBM5 transfer vector (AdBM5), the recombinant protein can accumulate at levels up to 10-20% of total cellular proteins (equivalent to 30–60 $\mu g/10^6$ cells), which makes it the most abundant protein in the infected cells. This yield compares advantageously to established expression systems, such as baculovirus/insect cell (Bac), for the production of non-secreted protein. Massie *et al.* (1994) compared the production of herpes simplex virus ribonucleotide reductase R1 and R2 subunits in both optimized culture of AV/293 and Bac systems. While the R2 subunit was about 5 fold more abundant and active in AV/293 than in Bac infected Sf9 cells, the R1 subunit was produced at roughly similar level in both systems. However, the amount of active soluble R1 obtained from AV/293 was at least 5 times higher than in Bac/Sf9 presumably due to better folding of the R1 protein in 293 cells. In terms of scale-up, the fact that, contrarily to Bac, AV virions remain concentrated within the cell long after yields have reached maximum levels, facilitating virus collection and concentration (Graham and Prevec, 1992) is also an advantage over Bac.

Another scale-up issue was the difficulty to design a large scale unit for the culture of adherent cells. The adaptation of the original 293A (anchorage-dependant) cells to suspension culture was a pre-requisite for the scale-up of the AV/293 system. The 293N3S subline developed by Graham (1987) by passage of the 293A cells through nude mice, was the first subclone of 293 cells successfully adapted to suspension culture. In our hands however, the 293N3S cells had a relatively long initial lag phase in suspension, a low growth rate, and a strong tendancy to clump, even in calcium-free medium. We then tested another subline, the 293S cells (Cold Spring Harbor Laboratories), obtained by gradual adaptation to suspension growth. The 293S cells grew more readily in suspension with no initial lag phase, a doubling time of 24 h and minimal clumping in calcium-free medium. Furthermore, 293S cells produced equivalent level of recombinant proteins compared to 293A (Massie *et al.*, 1994). The 293S cell line was therefore chosen for further process development.

Medium limitation and/or by-product inhibition is an important scale-up problem in animal cell culture in general and the AV/293 system is not an exception. Although 293S cells could reach plateau density of 2–5 $\times 10^6$ cell/mL depending on the culture medium, productive infection with AV was restricted to cell density lower than 5×10^5 cells/mL in batch culture without medium replacement. In this paper, we present a two-step approach, undertaken to improve the volumetric yield of the AdBM5/293S recombinant protein production system with the model protein Protein Tyrosine Phosphatase 1C (PTP1C). This 68kDa enzyme is a highly phosphorylated intracellular phosphatase that plays a crucial role in signal transduction and is a potential target for cancer therapy (Shen *et al.*, 1991). In the first step a medium replacement strategy has been applied, in order to rapidly overcome any medium-related limitation/inhibition problems. We will show the success and the limitations of this strategy. In a second step, an extensive metabolic analysis has been undertaken in order to identify more precisely what was limiting or inhibitory in the medium. This knowledge was then applied to a specific addition and control strategy of the infected culture. This longer procedure has lead to higher volumetric productivity with lower medium expenses.

Materials and methods

Cells, medium and virus

The 293A cells were used for plaque assay. The cells are derived from human kidney fibroblast transformed with Ad5 DNA and express the E1A and E1B proteins constitutively (Graham *et al.*, 1977). 293A were obtained from ATCC and sub-cultured twice weekly in DMEM with 10% fetal bovine serum in 25 cm^2 T-flasks. The 293S were obtained from Dr. Michael

Matthew (Cold Spring Harbor Laboratories). 293S were kept frozen in liquid nitrogen until used. A fresh cell aliquot was thawed every two months and maintained in 100 mL spinner flask at 37 °C, 5% CO_2 by diluting twice a week to cell densities 1–3×10^5 cells/mL with complete Joklik + medium (J +) described below.

J+ medium was inspired from Chillakuru *et al.* (1991) who used enriched DMEM for cultivation of vaccinia virus in HeLa cells. It was made of Joklik medium (calcium-free modification of MEM, Sigma) supplemented with 2.5 g/L glucose (total 4.5 g/L, 25mM, Sigma), 1X MEM essential amino acids (Gibco), IX MEM non-essential amino acids (Gibco), 1X MEM vitamin solution (Gibco), 0.11 g/L Na.pyruvate(Gibco), 5.7 g/L $NaHCO_3$ (Sigma) and 2.5 g/L HEPES buffer (Sigma). The mixture was then adjusted to pH=6.75 and filter-sterilized. Calcium-free DMEM (CFDMEM, custom made, Gibco) was also tested. This medium was supplemented to yield a final concentration of 4.5 g/L glucose and 0.11 Na.pyruvate, equivalent to J+. However, CFDMEM was different from J+ as it did not contain any other non-essential amino acids except serine (0.1mM in J+ vs 0.4mM in CFDMEM) and it's buffering capacity consisted in 3.7 g/L of $NaHCO_3$. Both media were always completed with 5% iron supplemented bovine calf serum (CS)(Hyclone) and 0.1% (w/v) pluronic F-68 (Gibco) unless otherwise stated.

The replication defective AV, AdBM5-PT, have been constructed in Dr Shen's laboratories, to produce protein tyrosine phosphatase (PTP1C)(Zhao *et al.*, 1993). A stock of the virus has been constituted and used throughout all of the experiments: 6×10^9 cells were infected at a multiplicity of infection (number of viruses/number of cells or MOI) of 1 and harvested 72 hours post-infection (hpi). The cell pellet was then diluted to 10^7 cells/mL with J+ and then freezed-thawed three times to liberate the virus. The stock has then been titrated to 1.2×10^9 pfu/mL by standard plaque assay method.

Culture and infection in spinner flasks

Unless otherwise stated, culture and production runs were done in 100 mL siliconized spinner flasks (Bellco) with 50 mL of cell suspension in a 37 °C, 5% CO_2, humidified incubator. Samples were taken on a daily basis for viable and total cell count and were kept at –80 °C for further analyses. Aliquots of 1×10^6 cells were centrifuged (13,000 g), the cell pellet was washed twice in PBS and then frozen at –80 °C.

Growth of 293S cells in batch cultures were initiated by inoculating fresh J + medium with $1–2\times10^5$ cell/mL in exponential growth phase.

Production runs were prepared by first centrifuging (600g, 15 min) aliquots of a cell culture in the exponential phase or in the very beginning of the plateau phase. To insure that the viral adsorption phase was identical for each assay, uniform conditions were imposed for the initial incubation of the cell/virus mixture. The cell pellets were then resuspended with the AdBM5-PT virus in either spent or fresh medium at a cell density of 10^7 cells/mL and a MOI of 10 to insure synchronous infection. These concentrated cell/virus suspensions were incubated 2 hours and then diluted at various cell densities with spent or fresh medium. The infected cultures were incubated 3–5 days while samples were taken once or twice daily. For medium replacement experiments, infected cultures were centrifuged at 600g for 15 min and the spent medium discarded and replaced with the same volume of fresh medium. In a few cases, pH was periodically adjusted (2–3 times/day) in spinner flasks by addition of 7.5% $NaHCO_3$ until the color of the culture returned to 7.1. In spinner flasks, pH was estimated by the medium color compared to standard flasks (red-orange at pH $\approx$ 7.1, yellow at pH $\leq$ 6.5).

Bioreactor description and operation

A 3.5 L bioreactor (Chemap CF-3000 with a CBC-10 control unit) was used with 2.7 L of culture volume in order to scale-up the spinner productions. The tank was equipped with 3 surface baffles to break the liquid surface and increase mass transfer. Mixing was performed with a marine impeller rotating at 100 RPM. The temperature was maintained at 37 °C with a water jacket. D.O. and pH probes (Ingold) were mounted for monitoring and control purposes. The pH was controlled at 7.0 by intermittent addition of 7.5% $NaHCO_3$ solution. Feed gas composition was regulated by the sequential opening of electro-valves: CO_2 was kept at 7% and O_2 set-point was under the control of dissolved oxygen (DO) in order to maintain DO above 20%. Operation parameters were sent to a Compaq Deskpro PC for data acquisition.

The bioreactor infection protocol was identical to the one used for spinner cultures except that transfer of fluid to and from the bioreactor was achieved through sterile connections instead of under a biological hood.

Analytical methods

Viable and total cells were counted on a haemacytometer. Viability was assessed by dye exclusion using erythrosine B. The 293S cells having the tendancy to agglomerate, special care was taken to separate the clumps without affecting viability.

Medium composition was analyzed via HPLC. The various amino acid concentrations were measured by a reversed phase method as described previously by Kamen *et al.* (1991). The glucose and organic acid concentrations were obtained using an Interaction Ion-300 organic acid column (Chemicals Inc) with 0.0033N sulphuric acid as a mobile phase and two detectors: a refractive index detector (model 410, Millipore) and a spectrophotometer detector (model 490, Millipore) at 210 nm.

The oxygen uptake rate was measured using a YSI model 53 biological oxygen monitor, following the protocol provided with the system.

SDS-PAGE electrophoresis of cellular proteins was performed as follows. Frozen cell samples were thawed and diluted to 10^7 cell/mL in extraction buffer (80 mM Tris-HCl pH 6.8, 2% (w/v) SDS and 10% (v/v) glycerol) and then sonicated (Heat Systems-Ultrasonics Inc, model W-375) 5s, 90W. Cell extracts (10 μL) were diluted with 10μL of NOVEX (San Diego, CA) sample buffer, containing 0,5% (v/v) β-mercaptoethanol. The diluted samples were heated at 85 °C for 5 min. and centrifuged 15s in an Eppendorf centrifuge before being loaded on a 8% acrylamide NOVEX precasted gel (10^5 cells per lane). The SDS-PAGE was run for 90 min. at 125 V following the NOVEX procedures.

Protein Tyrosine Phosphatase activity

PTP1C activity was measured according to the method described by Pot *et al.* (1991) with the following modifications. Batch analysis was performed by doing multiple dilutions in 96-well plates where samples were quantified against a PTP1C standard (kindly provided by Dr S.Shen). However, it was found that when diluted in the extraction buffer alone, the activity of the purified enzyme was drastically reduced. In order to stabilize the enzymatic activity, the purified PTP1C was diluted in a cell lysate obtained by adding 400 μL of the extraction buffer (described below) per 1×10^6 non-infected cell pellet. Samples, stored at –80 °C, were thawed on ice and resuspended at 2.5×10^6 cells/mL in extraction buffer: 25mM Tris-HCl, pH = 7.5, 10 mM β-mercaptoethanol, 2mM EDTA and 0.5% (v/v) Triton X-100. Aliquot volumes of 1 to 6 μL were transferred in the 96 well plate followed by 95μL of pNPP reagent: 25mM pNPP, 1.6mM DTT, 40 mM MES, pH=5. The plate was incubated at room temperature for 10 min and then 100 μL/well of 0.2N NaOH solution was added to stop the reaction. The plate was read at 405 nm using a Titertek Multiskan MCC microplate reader. A calibration curve was obtained from dilutions of the standard and the PTP1C content of the samples was calculated from that curve.

The specific PTP1C activity has been verified to be equivalent for cell samples and purified standard. For dilutions of identical activities, the PTP1C band obtained on PAGE for the purified standard was always equal or less than the PTP1C band for a cell sample. The stability of the frozen PTP1C standard was also assessed by series to series reproducibility of the activity calibration curve.

Yield (Y) and specific consumption rate (q) calculations

During growth experiments, the limits of the exponential growth phase were identified by first determining the zone of linear relationship on the plot of the natural log of cell concentration ln(X) vs time (t). The specific growth rate (μ) was then estimated as the slope of that ln(X) vs t plot and the doubling time (t_d) was computed: $t_d = \ln(2)/\mu$. The cellular yield per mole of consumed substrate (Y_s) was calculated by dividing the quantity of cells produced by the quantity of substrate consumed during the exponential growth phase, while the product per cell yield was the mole produced divided by the quantity of cell produced during the exponential growth phase. The specific substrate consumption (q_s) rate was obtained by using: $q_s = \mu / Y_s$.

During infection, since cells do not grow significantly, Y and q were calculated differently. Specific substrate consumption rates were estimated during the period of initial linear consumption by dividing the quantity consumed by the time interval and the mean cell concentration during that period. Product yield was obtained by dividing the maximum quantity produced by the total cell concentration at that time.

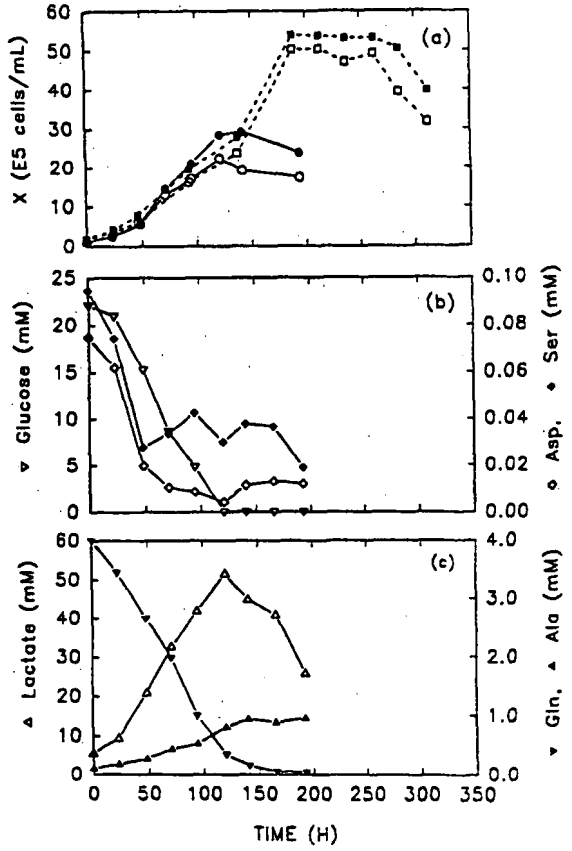

Fig. 1. Typical growth curves of 293S cells in Joklik+ and CFD-MEM. (a) o, • viable and total cells in J+, □, ■ viable and total cells in CFDMEM. Key metabolites profiles for cultures grown in Joklik+ medium: (b) ▽ glucose, ◊ aspartate, ♦ serine, and (c) △ lactate, ▼ glutamine, ▲ alanine.

Results and discussion

Growth kinetic of 293S cells in suspension culture

Typical growth curves for the 293S cells in J+ and CFDMEM, together with the variation of key medium components in J+ is shown in Fig. 1(a) through (c). Viable and total cell densities are shown in Fig.1(a). 293S cells were inoculated at 0.15×10^6 cells/ml in complete J+ medium. Exponential growth started soon after cell inoculation and was maintained for a period of 3 days with a doubling time of 24 h. The growth was then linear for the next two days followed, at day 5, by a plateau of $2–3 \times 10^6$ cells/mL. At that point, the viability of the culture did not decrease sharply but rather stayed at the plateau for a few days before declining.

As can be seen in Fig.1(b) and (c), the growth of 293S cells in J+ was characterized by a substantial consumption of: glucose ($Y_{glucose} 10^{11}$ cell/mole or $q_{glucose} = 29 \times 10^{-14}$ mole.(cell.h)$^{-1}$ in the exponential phase), aspartate ($Y_{asp} = 7.1 \times 10^{12}$ cell/mole or $q_{asp} = 0.4 \times 10^{-14}$ mole.(cell.h)$^{-1}$), serine ($Y_{ser} = 6.7 \times 10^{12}$ cell/mole or $q_{ser} = 0.43 \times 10^{-14}$ mole.(cell.h)$^{-1}$) and glutamine ($Y_{gln} = 5 \times 10^{11}$ cell/mole or $q_{gln} = 5.8 \times 10^{-14}$ mole.(cell.h)$^{-1}$). Although glucose and glutamine were consumed at a higher specific rate, aspartate and serine were depleted first since they are at a much lower concentration in the medium (0.1 mM compared to 25mM for glucose and 4mM for glutamine). Ammonia never exceeded 2mM at which concentration it was tested to be non-inhibiting for 293S growth (data not shown). On the other hand, lactate was the main by-product of the culture ($Y_{lact} = 2 \times 10^{-11}$ mole produced/cell) while a marginal amount of alanine was produced (1mM at day 6). Indeed, 293S cells did not oxidize significant amounts of glucose; most of it was used through glycolysis. Lactate has been found to impede 293S cell growth at concentrations of 20mM or more (data not shown). Consequently, lactate accumulation might have caused the shift from exponential to linear growth around day 3, although depletion of aspartate and serine may also be involved. Lactate accumulated to 50 mM by day 5, at which point glucose was depleted and the cells stopped dividing. Glucose and/or glutamine depletion around day 5 was most probably the cause of the culture entering the plateau phase, after which the cells started consuming lactate.

The 293S cells were also grown in complete CFD-MEM (Fig.1(a)). As can be seen, while the growth kinetics were equivalent in both media up to day 5, beyond that point, cells in CFDMEM kept growing for another 3 days reaching a cell density of 5×10^6 cells/mL. In contrast, cells in J+ entered the plateau phase at this time. A 3 day plateau then occurred before the cell density started decreasing. As described in the previous section, the only difference between CFD-MEM and J+ is the lower buffering capacity and the absence of non-essential amino acids with the exception of serine which is four times more concentrated. The need for higher levels of serine and/or the effect of the buffers (especially HEPES) on cell/substrate yields (glucose or glutamine) could explain the better performance of CFDMEM for 293S cell growth.

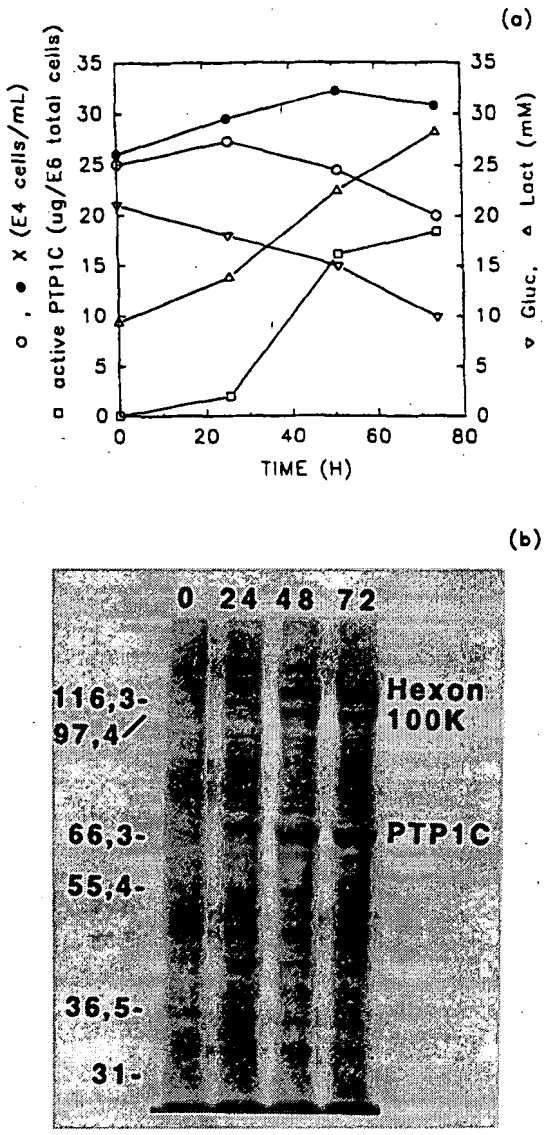

Fig. 2. Typical batch production of PTP1C at low cell density (0.25×10^6 cells/mL) in Joklik+ o, • viable and total cells, □ PTP1C, ▽ glucose, △ lactate; (b) SDS-PAGE of cell extracts sampled at different time the infection, 10^5 cells per lane: 0, 24, 48 and 72 hpi.

Production of PTP1C in AdBM5-PT infected 293S at low cell density

Fig. 2(a) shows the increase in cell density with time for infecfed 293S cells seeded at 0.25×10^6 cells/mL. The active PTP1C cell content, glucose consumption as well as the lactate accumulation are also shown. The growth rate for infected cells was close to zero and cell viability fell gradually during the 3 day production phase. This is typical of a viral infection where the virus utilizes the host's cellular machinery towards the production of it's own DNA, RNA and proteins, thus impeding cellular growth and eventually causing cell death. Active PTP1C was produced at a constant rate and reached a level of 18 $\mu g/10^6$ cells 3 days post-infection. Furthermore, as can be seen by SDS-PAGE of the cell extract (Fig.2b) for samples at 0, 24, 48 and 72 hpi, the PTP1C band at 68kDa followed the same accumulation kinetics as the activity assay. It can also be seen in Fig.2(b) that PTP1C constituted the most abundant cellular protein, overtaking the hexon and 100K viral proteins.

As for the metabolites' evolution, glucose consumption as well as lactate accumulation were significant but not limiting the culture (Fig.2a); the other components in the medium were also not limiting (data not shown). This was expected since at low cell density infections, conditions are optimal for production because no limitations and/or inhibitions take place. However, specific consumption rates for glucose, 46×10^{-14} mole.(cell.h)$^{-1}$ and glutamine, 9.4×10^{-14} mole.(cell.h)$^{-1}$ were 60% higher than during growth while the rates for aspartate and serine, q= 0.25×10^{-14} mole.(cell.h)$^{-1}$ decreased by 40% with respect to growth. The significant increase in glucose and glutamine consumption rate, both primary sources of energy, indicate a general acceleration of the cell metabolism during infection. Once again, lactate accumulated at a specific rate twice that of glucose consumption (1×10^{-12} mole.(cell.h)$^{-1}$) implying complete glycolysis.

Production of PTP1C at higher cell densities: effect of medium replacement

A first series of experiments was undertaken in order to precisely establish the potential of the production medium in terms of the maximum cell density at infection that would not impair product yield. 293S cells were infected at different days in a culture and therefore at different densities (day 2, 0.6×10^6, day 3, 1.3×10^6 and day 4, 1.7×10^6 cells/mL) have been infected and resuspended at their initial cell densities in either (a) their spent medium, (b) fresh medium or (c) fresh medium followed with a medium replacement at 24 hpi. The resulting PTP1C yields at various time points are presented in Fig. 3 (a) through (c) and compared to a culture control infected a low cell density (0.25×10^6 cells/mL). It can be seen in Fig. 3(a) that at 0.6×10^6

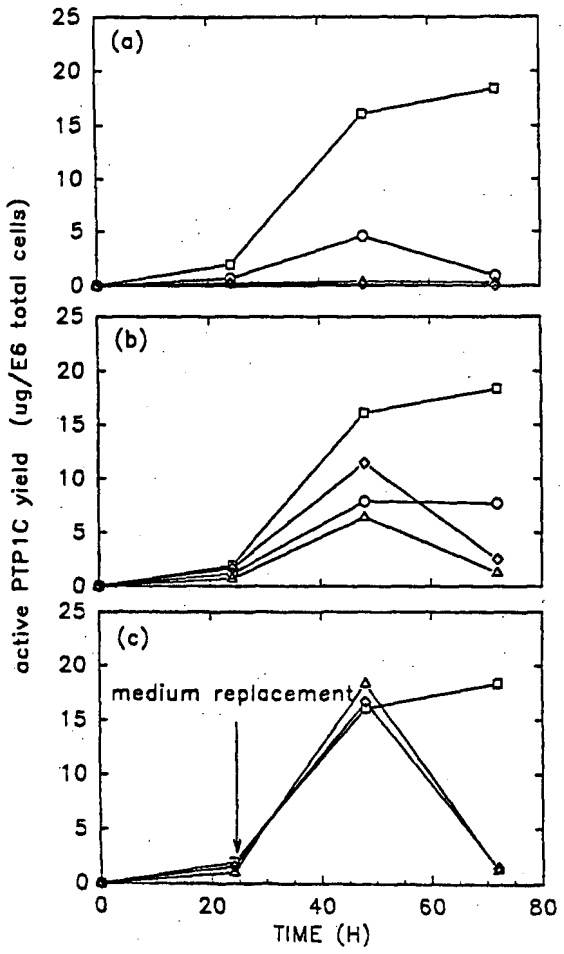

Fig. 3. PTP1C production in J+ at different cell densities. Infected cells resuspended in: (a) spent medium, (b) fresh medium, and (c) fresh medium with medium replacement at 24 hpi. Cell densities at infection: o 0.6×10^6, ◊ 1.3×10^6 and △ 1.7×10^6 cells/mL. □ duplicate controls at 0.25×10^6 cells/mL resuspended in spent or fresh medium.

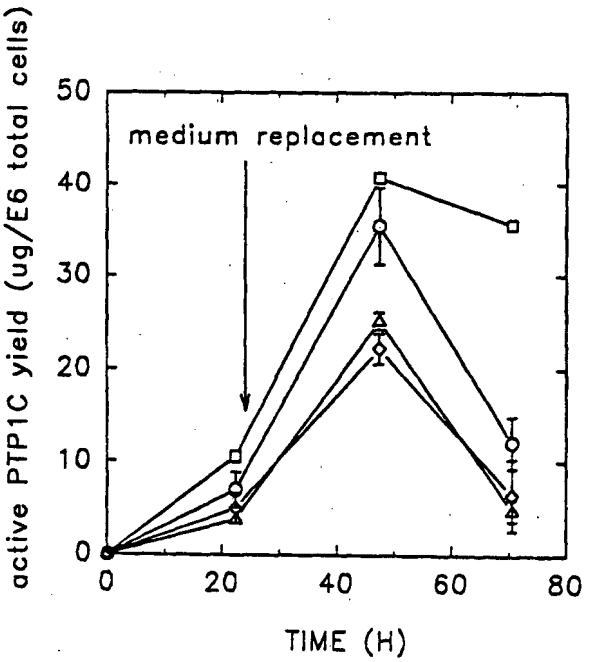

Fig. 4. PTP1C production in fresh J+ at different cell densities with one medium replacement at 24 hpi. o 2×10^6, ◊ 3×10^6, △ 4×10^6 cells/mL. □ control at 3×10^5 (without medium replacement). Means of duplicates are represented (± S.D.).

cells/mL or above, no significant amount of PTP1C was produced when the infected cells were resuspended in their spent medium. However, when resuspended in fresh medium (Fig.3b) production regained 50% of the maximum productivity with respect to the control at all cell densities. Furthermore, a second medium replacement at 24 hpi (Fig.3c) allowed for a sustained maximum specific productivity, even at the highest cell density (initial 1.7×10^6 led to a final 2.2×10^6 cells/mL).

These results clearly establish the existence of a substrate limitation and/or a by-product inhibition at high cell densities, a problem which can be partially remediated by an initial cell resuspension in fresh medium (Fig.3b) and completely restored with a medium change at 24 hpi (Fig.3c), resulting in a maximum productivity comparable to optimal low cell density infection. It also establishes that the cell culture growth stage (from early exponential at day 1 to beginning of plateau phase at day 4) do not influence protein production since cells infected in their 4th day of culture produced as much PTP1C as one day old infected culture, as long as the medium is not limiting and/or inhibiting. However, this medium replacement strategy was apparently only effective for a period of 24 to 48 h since the PTP1C activity decreased abruptly at 72 hpi. Analysis of total cell protein by SDS-PAGE showed that the loss in activity at 72 hpi was not concurrent with an equivalent loss in total PTP1C content (data not shown). The decrease in PTP1C activity ohserved at 72 hpi was therefore not due to protein degradation, but rather to an unknown mechanism such as protein aggregation, as previously observed for the HSV R1 subunit expressed with a similar AV (Massie *et al.*, 1994) or a major change in the phosphorylation state of the protein. Resolving this issue will require further investigation.

In order to evaluate the limit of this initial and 24 hpi medium replacement strategy, PTP1C yield was tested following infection of 293S cells at 2, 3 and

4×10^6 cells/mL. As presented in Fig. 4, at 48 hpi, the yield of PTP1C for infection at 2×10^6 cells/mL (35 ± 4.3 $\mu g/10^6$ cells) was not significantly different from the control ($40\mu g/10^6$ cells), while for higher cell densities yields were 40% inferior to the control. Past 48 hpi, the active product yield fell to zero for all the experiments at high cell densities while the low density control remained constant. These results show that 2×10^6 cells/mL is the maximum cell density at which daily medium replacement with J+ medium allows for the maintenance of maximum specific productivity. At cell densities higher than 2×10^6 cells/mL, volumetric productivity as well as specific product yield per cell decreases thereby increasing production and purification costs.

Production of PTP1C at high cell density in a 3L bioreactor

Since CFDMEM was more efficient for 293S growth than J+, its performance during an infection was tested. However, a preliminary experiment comparing both media for an infection at high cell density showed that CFDMEM acidified more rapidly than J+, which was not the case during cellular growth. At 72 hpi, in cell cultures infected at 1.3×10^6 cells/mL in fresh medium with a medium replacement at 24 hpi, the pH dropped below 6.5 (yellow medium) in CFDMEM while pH was roughly equal to 6.8 (orange medium) in J+ (data not shown). As a result, the PTP1C activity was also lower in CFDMEM (20 $\mu g/10^6$ cells) than in J+ (32 $\mu g/10^6$ cells) at 48 hpi.

In order to assess the correlation between active PTP1C production and pH as well as the scalability of the process, an infection experiment was performed in a pH-controlled 3L Chemap bioreactor. The results are shown in Figure 5 for a culture infected at 2×10^6 cells/mL (MOI=10) in CFDMEM. The bioreactor was compared to infected cell controls: two 50mL spinner flasks initially taken from the bioreactor, one with and the other without periodical pH adjustment.

The PTP1C specific productivity was equivalent in the bioreactor and in the pH controlled spinner with a peak of 45 μg active PTP1C/10^6 cells at 48 hpi, followed by a slight decrease in the active PTP1C concentration. By contrast, in the spinner flask without pH control, the accumulation of active PTP1C stopped at 30 hpi with a peak of 30 μg active PTP1C/10^6 cells, followed by a rapid reduction in activity, falling close to zero by 52 hpi. A correlation between the PTP1C activity loss and pH decrease was observed. While at

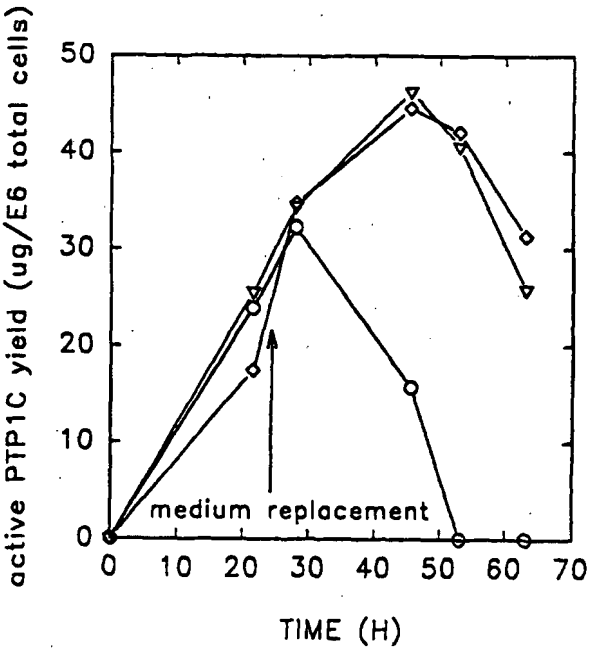

Fig. 5. Production of PTP1C in bioreactor at 2×10^6 cells/mL with fresh CFDMEM and a medium replacement at 24 hpi. ◊ bioreactor, ∇ spinner control with periodical pH adjustments, o spinner control without pH adjustments.

48 hpi the medium was already yellow in the non-controlled spinner (pH $\leq$ 6.5), the medium was still red-orange in the bioreactor and the pH adjusted spinner (pH $\approx$ 7). However, the decrease in pH does not fully explain the PTP1C activity loss since the PTP1C activity also decreased slowly in a pH controlled environment past 48 hpi. The pH control only delays the lytic process that inevitably takes place during adenoviral infection, while permitting the maximum product yield to be attained.

These results show that it is possible to maintain maximum specific production rate at high cell densities, by medium replacement at 0 and at 24 hpi, in a pH controlled culture at the 50 mL spinner flask scale as well as 3L bioreactor scale. Although peak product yield varied among the experimental runs, one can expect to obtain 40 to 45 μg PTP1C/10^6 cells, equivalent to 15% of the total cellular protein content (based on 300μg total protein/10^6 cells) or 90 mg PTP1C per Litre of culture (at 2×10^6 cells/mL) compared to 13.5 mg/l for 0.3×10^6 cells/mL. These figures are comparable to productions of Herpes Simplex Virus ribonucleotide reductase subunits R1 and R2 obtained previously in our lab with other AdBM5 AV's (Massie *et al*, 1994). Although this process is very effective with respect to cell yield and protein purification for

an intracellular product, it is not optimal in terms of medium expenses. In fact, with two medium changes, the yield of product per spent medium is equivalent to 30 mg PTP1C per L of medium (at 2×10^6 cells/mL) which is not much higher than 15 mg/L of medium for productions at 3×10^5 cells/mL without medium replacement. We then turned our attention to the analysis of key metabolites in order to improve yield based on spent medium.

Analysis of key metabolites during the infection phase

Samples from the infection of 1.3×10^6 293S cells/mL in fresh J+ medium without medium replacement at 24 hpi (for which PTP1C yield has been presented in Fig.3b) have been analyzed for their content in glucose, organic acids, and amino acids. In this experiment, PTP1C was only 60% the level of it's maximum specific activity due to limitation of nutrients and/or inhibition of by-products. The results are presented in Fig. 6.

The total cell density increased from 1.3×10^6 to a mean value of 1.9×10^6 cells/mL during the first 24 h and remained constant thereafter. This slight initial increase of about 20% in cell count is routinely observed for infected culture. However, the maintenance of viability up to 96 hpi is peculiar to infection in limiting and/or inhibiting environment. This could be explained by the fact that under sub-optimal conditions of infection, the overall cycle of virus reproduction would occur at a lower rate, thereby reducing the infection stress on the cell which in turn would result in a prolonged viability.

Glucose was completely depleted before 48 hpi. Based on the first 24 hour period, the specific glucose consumption rate, was 38×10^{-14} mole glucose.(cell.h)$^{-1}$ which is a 30% increase compared to $q_{glucose}$ during cell growth. However, glucose consumption rate was reduced by 17% compared to infection at low cell density. Furthermore, while glucose was totally transformed into lactate during growth and infection at low cell density, only 45% of it was metabolized through glycolysis during the infection phase at high cell density; 22mM of glucose yielding only 20mM of lactate instead of 44mM for a complete glycolysis. It appears that while the general metabolic activity was higher compared to growth phase, in this case there was a glucose limitation, thereby reducing glucose consumption rate as well as lactate production rate. This in turn should increase the cellular oxygen requirement. The specific oxygen consumption rate

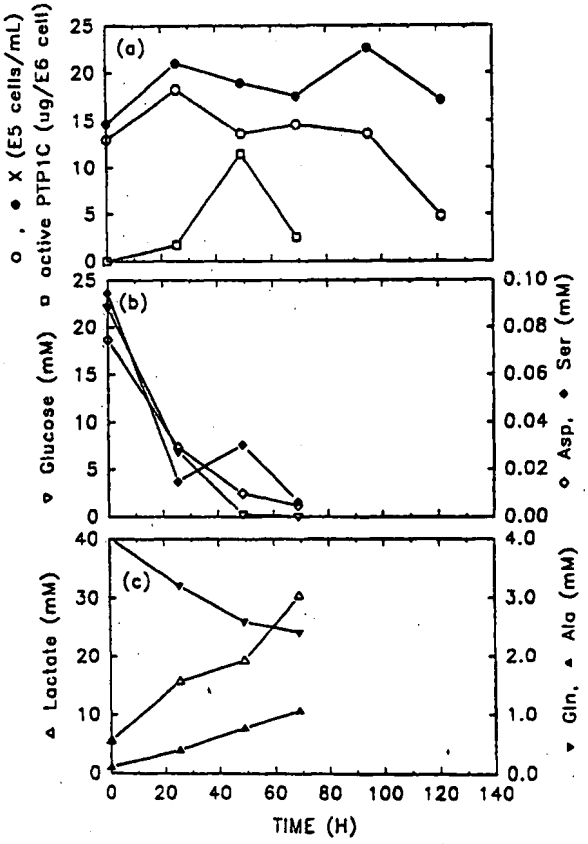

Fig. 6. Key metabolites evolution in a batch production of PTP1C in fresh J+ at initial cell density of 1.3×10^6 cells/mL: a)o, • viable and total cells in J+, □ PTP1C yield; (b) ▽ glucose, ◇ aspartate, ♦ serine, and (c) △ lactate, ▼ glutamine, ▲ alanine.

(q_{O2}) has been measured in growth phase as well as during infection. Indeed, the average value obtained during infection, $q_{O2} = 16\times10^{-14}$ mole O_2.(cell.h)$^{-1}$ was twice as high as for exponential growth, $q_{O2} = 8\times10^{-14}$ mole O_2.(cell.h)$^{-1}$. This is consistent with a significant increase in metabolic rate during the infection phase compared to the growth phase and a reduced glycolysis rate with respect to nonlimiting medium conditions.

During infection, aspartate and serine specific consumption rates were low ($\approx 0.2\times10^{-14}$ mole.(cell.h)$^{-1}$ for both), but, given the high cell density, were rapidly depleted by 48 hpi. Glutamine was consumed (2.2×10^{-14} mole.(cell.h)$^{-1}$) but not depleted and alanine was produced (1×10^{-14} mole.(cell.h)$^{-1}$). These specific consumption rates were smaller than those obtained for infection at low cell density and exponential growth. This might be due to glucose limitation. However, aspartate and serine, although depleted, were not limiting. Indeed, compar-

isons of PTP1C production in J+ and CFDMEM at high cell densities were not found to be significantly different, even though CFDMEM did not contain aspartate but four times the serine concentration of J+. In other production runs, 6mM glutamine (instead of 4mM) as well as 10% serum addition (instead of 5%) have also been tested, but did not give higher productivities (results not shown). However, a 50% drop in PTP1C production was observed in absence of serum compared to the usual 5% CS.

In summary, in serum supplemented culture, the production of PTP1C with the AdBM5/293S system in CFDMEM was mainly limited by glucose depletion and inhibited by the pH drop caused by lactate accumulation.

Effect of specific additions during a production run

In order to apply and verify the above results, PTP1C production was compared in a glucose addition vs a medium replacement experiment. A culture at 1.5×10^6 cells/mL was infected, resuspended in fresh CFDMEM with 2.5 g/L HEPES and aliquoted into three 50mL spinner flasks. In the first spinner the culture was centrifuged and the medium replaced with fresh CFDMEM + HEPES at 24 hpi. In the other two, 0.5 mL of a 200 g/L glucose solution was added to the culture at 24 hpi (+2 g/L, or 11 mM glucose addition). In one of these pH was periodically adjusted.

Figure 7 shows that, PTP1C production followed a similar profile in both the medium replacement and the glucose addition experiment where pH was controlled. The two feeding strategies yielded a maximum active PTP1C content of 25 $\mu g/10^6$ cells at 36–48 hpi. Since it has been shown that at high cell density without medium replacement or glucose addition, active PTP1C yield declined after 24 hpi, it is clear that glucose addition is responsible for sustained PTP1C production, equivalent to production with medium replacement. It therefore confirms that glucose is most probably the major limiting substrate of PTP1C production.

In the third spinner (glucose addition without pH control) the PTP1C activity decreased linearly after 24 hpi and was absent by 48 hpi. This severe drop in activity is again correlated with a decrease in pH; indeed, the medium was already yellow at 36 hpi or earlier ($pH \leq 6.5$) in the glucose addition spinner without pH control while it was maintained around red-orange ($pH \approx 7$) in the other spinner with glucose addition. The exclusive relation between pH and PTP1C activity

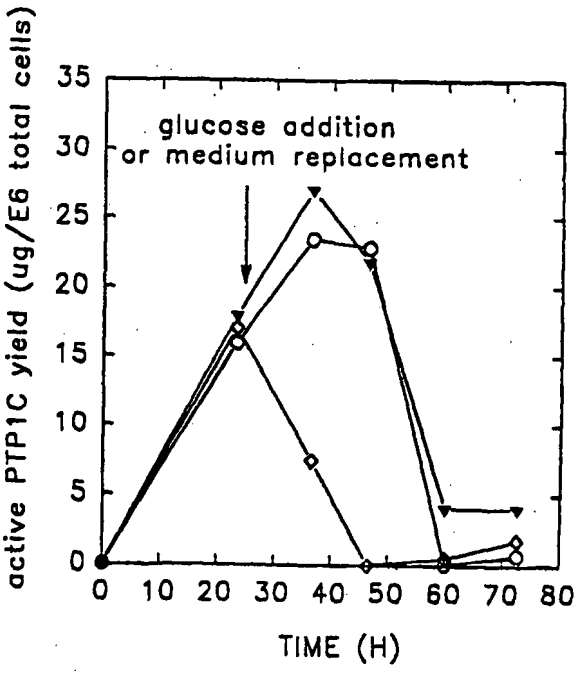

Fig. 7. Production of PTP1C in fresh CFDMEM at 1.6×10^6 cells/mL. o medium replacement at 24 hpi, ▼ glucose addition with periodical pH adjustments, ◊ glucose addition without pH control.

was confirmed by lactate analysis since lactate concentrations attained equivalent level (40–50 mM) in both glucose-supplemented spinners (data not shown). It is therefore the pH decrease as such and not lactate production, that has a negative effect on active PTP1C yield. This is encouraging since pH is easier to control than lactate production.

Conclusion

In this paper we have presented the first results concerning the scale-up of a high-level recombinant protein production AV/293 system. The 293S cells have been shown to be able to grow to plateau cell densities of 5×10^6 cells/mL in calcium-free DMEM. With an initial and a 24 hpi medium replacement, the specific PTP1C yield could be maintained at it's maximum level up to infected cell densities of 2×10^6 cells/mL. Under these conditions, volumetric productivities of 90 mg/L could be attained. At an infected cell density of 1.6×10^6 cells/mL, the replacement of the 24 hpi medium change by a 2 g/L glucose addition, together with periodical pH adjustments, allowed the same specific productivities, but at lower medium expenses.

It is expected that glucose fed-batch in pH-controlled bioreactor will further improve these performances.

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Exhibit 3

Improved Adenovirus Vector Provides Herpes Simplex Virus Ribonucleotide Reductase R1 and R2 Subunits Very Efficiently

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We have constructed a new adenovirus (Ad) expression vector, pAdBM5, that allows for the production of unprecedented levels of recombinant protein in the human 293 cell line using the Ad expression system. The main feature of this vector is a combination of enhancer sequences that increases the activity of the ectopic major late promoter (MLP) in recombinant Ad. In 293 cells infected with helper-free Ad recombinants generated with the pAdBM5 transfer vector, both herpes simplex virus (HSV) ribonucleotide reductase R1 and R2 subunits represent the most abundant polypeptides, accounting for as much as 15–20% of total cellular proteins. Our data suggest that this level of expression is probably very close to the upper limit of the system. Furthermore, when compared to the widely utilized baculovirus (Bac)/Sf9 expression system, the improved Ad vector showed a better performance for the production and purification of active HSV-2 ribonucleotide reductase R1 and R2 subunits. The R2 subunit was about 5-fold more abundant in recombinant Ad-infected 293 cells than in Bac-infected Sf9 cells while the R1 subunit was produced at roughly similar levels with either system. However, the amount of active soluble R1 obtained from recombinant Ad-infected 293 cells was at least 5 times higher because most of the R1 produced in Sf9 cells was insoluble.

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Since their development in the early '80s, Ad vectors have been successfully used to express a wide variety of viral and cellular genes for three main applications: (1) high level expression of heterologous proteins, (2) live viral subunit vaccines, and (3) gene transfer vectors for establishing stable cell lines or for gene therapy (reviewed in refs. 1–4). Ad were initially considered as good candidates for high level expression of recombinant proteins for a number of reasons. First, human Ad can replicate efficiently to very high titer (10^8–10^9 pfu/ml) in human cells as well as in other mammalian cells, secondly they produce their late proteins at levels of 30–40% of total cellular proteins (TCP), and finally they can be propagated in suspension cultures. The high level of expression of the abundant late proteins is the result of the strong transcriptional activity of one promoter, the MLP, which is responsible for the production of some 20 late mRNAs. These mRNAs are all derived from one long primary transcript by maturation processes involving differential splicing and polyadenylation events. Three structural proteins, namely hexon (15–20% of TCP), fiber (8–10%), and penton (2–4%), and one non-structural protein, named 100K (5–10%), represent the major proteins in Ad-infected cells[5,6].

The construction of helper-independent-defective Ad recombinants is generally accomplished by cloning expression cassettes in the deleted E1 region. By deleting the E1 and E3 regions of the Ad genome, expression cassettes of up to 7–8 kbp can be introduced in Ad[7,8]. While the E3 functions would mainly be involved in down regulating the host immune response to Ad infection and are totally dispensable for Ad replication in tissue culture (reviewed in ref. 3), the deletion of the E1 region makes Ad replication defective in all mammalian cells except complementing cell lines such as 293 cells which constitutively express the Ad E1 polypeptides[9]. So far, most of the helper-independent-defective Ad recombinants that express foreign proteins at high levels were constructed using MLP-based expression cassettes inserted into the Ad genome of the serotype 5 (reviewed in refs. 1 and 3). The ectopic MLP sequences found in those expression cassettes generally incorporate between 200 and 700 bp upstream, and 33 bp downstream of the transcriptional start site[2]. Those sequences were expected to contain all of the required elements that confer full transcriptional activity to the endogenous MLP. However, the majority of recombinants constructed thus far express levels of heterologous proteins that are usually much lower than the levels of the most abundant Ad late proteins[10–20]. Examples of the best recombinants include AdSVR112 (refs. 7, 21) which expresses the SV40 large T antigen at 3–4% of TCP, and Ad5-RR_2^{HSV}[22] which expresses the HSV-2 ribonucleotide reductase R2 subunit at 4–5% of TCP. Also reported are Ad recombinants that appear to produce foreign proteins at levels slightly higher than 5% of TCP, although no accurate quantification was presented in those cases[23–25]. As well, yields of 2–3% of TCP were reported for the expression of HBsAg using Ad recombinants derived from the serotypes 4 and 7 (ref. 26). Thus, none of the Ad recombinants described so far have expressed their heterologous protein at a level equivalent to, or higher than the level of hexon or 100K, the two most abundant proteins in Ad-infected cells.

In the transfer vector pAdBM1, one of the best recombinant cistrons assembled so far for high level of expression[22], and in similar vectors, the expression cassette include sequentially: the strong MLP, a highly efficient translational leader (Ad2 tripartite leader), splicing signals, and one or multiple polyadenylation sites (Fig. 1A). Given that, in such vectors, the ectopic MLP drives the expression of only one mRNA whereas the endogenous MLP produces more than 20 mRNAs, one would have expected that the expression level of the recombinant protein could equal at least half of the sum of the late viral proteins. Since this has not been observed, one likely explanation is that the ectopic MLP lacked sequences required for its full transcriptional activity.

Here we report on the construction of a new Ad expression transfer vector, pAdBM5, in which enhancer sequences have been introduced to further stimulate the transcriptional activity of the ectopic MLP. Using the HSV-2 ribonucleotide reductase R1 and R2 subunit genes, we show that in human 293 cells infected with helper-free Ad recombinants generated with pAdBM5, the recombinant protein can represent the most abundant polypeptide. Furthermore, this improved Ad expression system is more efficient than the widely utilized Bac sys-

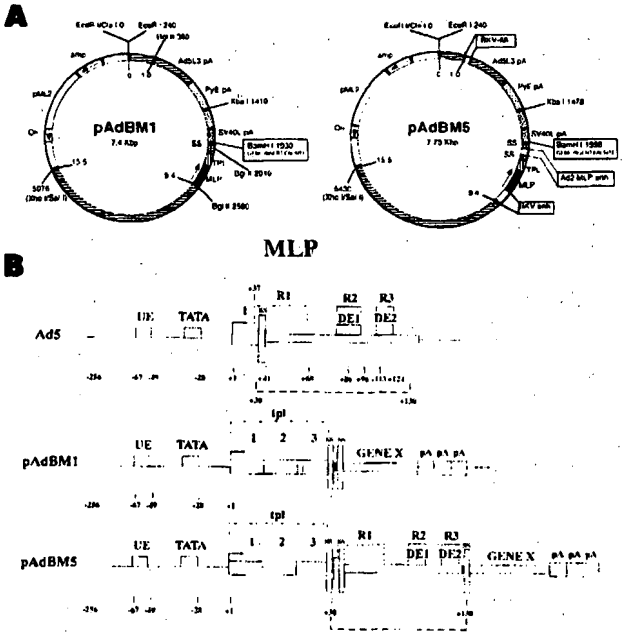

FIGURE 1. (A) Genetic maps of pAdBM1 and pAdBM5 transfer vectors. All of the genetic elements present in pAdBM1 have been described in details in Lamarche et al.[18]. As illustrated on the diagrams, pAdBM5 was derived from pAdBM1 by successive cloning of BKV enhancer elements at Bgl II sites nucleotides 380 and 2590, and Ad2 MLP "enhancer-like" element at Bgl II site nucleotide 2010 on pAdBM1 map as detailed in Experimental Protocol. The inner number on the vector refer to map unit (m.u.) position on the Ad5 genome. pML2 is the *E. coli* replicon; the segments with dashed lines (0–1, and 9.4–15.5 m.u.) bracketing the MLP-based expression cassette (between 1.0 and 9.4 m.u.), are Ad5 subgenomic portions involved homologous recombination to generate Ad recombinant and in Ad replication. (B) Diagrams of Ad5 endogenous MLP DNA fragment as found in its normal location in the genome or ectopic MLP DNA fragments as found in Ad transfer vectors. (See text for details). The diagrams are not drawn to scale. Symbols: (SS) splicing signal, (tpl) tripartite leader, (pA) polyadenylation signal.

tem for the production and purification of active HSV-2 R1 and R2 proteins.

Results

Construction of the transfer vector pAdBM5 and of recombinant Ad. The objective of the manipulations performed on pAdBM1 to obtain pAdBM5 was to improve the productivity of the Ad recombinants by increasing the transcriptional activity of the ectopic MLP. The human Ad MLP is one of the strongest mammalian promoters known[27]. Although active in the early phase of the infection, its transcriptional activity is increased 30–50 fold during the late phase. Numerous *cis*-acting sequences are essential to confer full transcriptional activity to the MLP[28–30]. These include an upstream element (UE) between –67 and –49 relative to the transcriptional start site, a TATA box centered at –28, and an initiator element encompassing the transcription start site. In addition, some downstream elements (DE) have been mapped and designated, R1 (+37 to +68), DE1 (+85 to +96) or R2 (+80 to +105), and DE2 (+109 to +124) or R3 (+105 to +125) (Fig. 1B). While the UE, the TATA box and the R1 downstream element have been shown to be important for basal transcriptional activity of the MLP both at early and late times, DE1 and DE2 are essential for late phase specific activation. At this point, it is not clear whether these *cis*-acting sequences could be moved from their normal position while maintaining their transcriptional activity. In any event, the fact that they are missing in all but one of the MLP currently used in Ad transfer vectors could explain in part the limited success obtained with the previously reported Ad recombinants.

To increase the transcriptional activity of the ectopic MLP, the DE (R1, R2, and R3), from +30 to +130 (ref. 28), were cloned into the intron lying between the third segment of the Ad tripartite leader and the Bam HI site as depicted in Figure 1. Thus in pAdBM5, these *cis*-acting elements have been displaced some 210 bp further downstream relative to their position in the endogenous MLP where they are located within the intron between the first and the second segment of the tripartite leader (Fig. 1B). In addition, the human BKV enhancer, which has been shown to activate the Ad MLP in several cell lines and more efficiently in 293 (ref. 31), was inserted at position –256 relative to the MLP transcription start site (Fig. 1A).

The effect of these modifications was tested with the HSV-2 R1 and R2 ribonucleotide reductase subunit genes, which had already been inserted in pAdBM1 (refs. 22, 32). With these new constructs, we hoped to facilitate the purification of large quantities of these proteins for biochemical and structural studies[32–34]. Ad5BM5-R2, and Ad5BM5-R1 were generated essentially as their BM1 counterparts. The genomes of all those recombinants are approximately the same size as wild-type Ad5, and are stable after at least 10 rounds of amplification, which is sufficient to produce large stocks of viruses or recombinant proteins. Whereas Ad5BM1-R2 and Ad5BM1-R1 grew routinely to titers only slightly lower than that of their parental virus, Ad5/ΔE1ΔE3, the growth of the two BM5 recombinants was reduced 5- to 10-fold.

Expression of HSV-2 R2 and R1 with pAdBM1- and pAdBM5-derived recombinants. As previously reported for Ad5BM1-R2 (ref. 22), the synthesis of recombinant R2 in Ad5BM5-R2 infected 293 cells follows a pattern similar to that of the Ad late proteins, where synthesis begins 12 hours post-infection, rapidly increases by 16 hours, and remains elevated until 30 hours post-infection (data not shown). The time course of recombinant R2 accumulation in Ad5BM1-R2 and Ad5BM5-R2-infected 293 cells was analyzed with Coomassie blue stained gels. As shown in Figure 2A, the R2 protein synthesized in Ad5BM5-R2-infected 293 cells accumulated during the late phase of infection at significantly higher levels than in Ad5BM1-R2-infected 293 cells. Thus, with Ad5BM5-R2-infected cells, R2 was clearly the most abundant protein by 30 hours post-infection and its level remained constant thereafter. Altogether, these results suggested that the higher level of R2 accumulation with Ad5BM5-R2 was most likely due to a higher rate of synthesis of the recombinant protein, presumably resulting from an increased rate of transcription of the R2 gene. Precise quantification of the R2 accumulation by densitometric analysis of Coomassie blue stained gels with purified R2 as standard gave, for several protein productions, levels within the range of 4–6% for Ad5BM1-R2 and 11–15% for Ad5BM5-R2 (data not shown). Therefore, the expression cassette modifications improved the recombinant protein expression by a factor of three.

For HSV-2 R1, the other ribonucleotide reductase subunit, the relative expression efficiency of pAdBM1- and pAdBM5-derived recombinants was estimated mainly from Coomassie blue stained gels as the one presented in Figure 2B. At the peak of protein accumulation which last from 30 to 48 hours post-infection for both vectors, a 3-fold increase was obtained with the pAdBM5 derived-recombinant. Quantification using purified R1 protein as a standard yielded, for several productions, levels of expression within the range of 4–6% of TCP for Ad5BM1-R1 and 12–20% for Ad5BM5-R1. We also compared the level of expression of HSV-2 R1 obtained with

Ad5BM1-R1 to that obtained with the previously published recombinant, AdRed-1, which uses a similar MLP-based vector to express the HSV-2 R1 protein with the exception of the polyadenylation sequences and of the orientation of transcription of the expression cassette[15]. The R1 produced by AdRed-1, which was not detectable on Coomassie blue stained gel (Fig. 2B, lane 2) was evaluated by immunoblotting to be 8-fold less abundant than with Ad5BM1-R1 (data not shown). Since the main difference between these two constructs is the inclusion of three polyadenylation sites in Ad5BM1-R1, this result emphasizes the importance of efficient polyadenylation for high-level expression of recombinant protein in MLP-based vectors.

From the gels performed to quantify the recombinant R2 and R1 expression, another important difference between the pAdBM1- and pAdBM5-derived recombinants was noticed. Whereas in cells infected with pAdBM1-derived recombinants the amount of the hexon was only slightly reduced compared to that obtained with the parental virus Ad5/ΔE1ΔE3, it was further reduced with pAdBM5-derived recombinants. Taken together with the proportional reduction in the titers of the respective recombinant Ad mentioned above, the reduction in the level of hexon suggests that the synthesis of the recombinant protein takes place at the expense of the Ad abundant late proteins.

Scale-up of the Ad expression system using 293S in suspension cultures. As suspension cultures are the overwhelming choice for large-scale production processes with animal cells, we tested the ability of the 293S cells, a 293 cell line adapted to grow in suspension in Joklik's medium (without Ca^{++}), to produce R1 and R2 upon infection with the improved recombinants. The first evaluation was done by comparing the production potential of 293S and 293A (adherent) cells grown in monolayer culture with either DMEM+10% FBS or Joklik+5% CS. Quantification of several Coomassie blue stained gels, as the one shown in Figure 3, indicated that no major difference could be found with either cell line or media for the production of Ad late proteins or recombinant R1 and R2.

Although 293S cells tend to form aggregates when grown in suspension culture, they are easy to culture since they can be diluted as low as 5×10^4 cells/ml, and routinely reach more than 2×10^6 viable cells/ml in a few days. When spinner cultures of 293S were used to produce virus stocks and recombinant R1 and R2, they performed as well as large culture of 293A cells in roller bottles. Moreover, under optimal conditions of infection, as detailed elsewhere[35], maximum recombinant protein yield can be maintained at cell densities as high as 2×10^6 viable cells/ml, resulting in reductase subunit production routinely around 90 mg/l.

Comparison of Ad and Bac expression systems for the production and purification of HSV-2 R2 and R1. The Bac/Sf9 expression vector system (BEVS) is known to be very efficient for high level production of recombinant proteins in animal cells (reviewed in ref. 36). Before the improvement of the Ad expression system, we had anticipated that the BEVS, for which we had already developed the procedure for efficient scale-up in bioreactors[37,38], could be more useful than the Ad system to produce large quantities of HSV-2 R1 and R2 subunits. To test this possibility, we used the best available Bac transfer vectors to insert into the genome of AcNPV the HSV-2 R1 and R2 genes under the control of the strong polyhedrin promoter to generate the recombinants Bac-R1 and Bac-R2. When the level of expression of recombinant R2 was measured in Sf9 cells infected by two different plaque isolates of Bac-R2, it was found to be roughly 2-fold lower than in Ad5BM1-R2-infected 293 cells (data not shown). This was surprising since the Bac transfer vector used to construct Bac-R2 was similar to the optimized vectors pAcYM1 and pAcRp23 (ref. 36), that we had already used to express the bovine rotavirus VP6 protein at a level approaching the level of polyhedrin (20% of TCP)[37,38]. The possibility that our Bac-R2 clones were not completely pure, or that we could have picked bad plaque isolates in our initial screening, was ruled out by the observation of similar low levels of R2 expression after additional rounds of plaque purification on these two clones and on another independent plaque isolate (data not shown).

To evaluate more precisely the performance of the two systems, the R2 protein was purified from several liters of suspension cultures of either Sf9 or 293S infected cells using a protocol developed by Lankinen et al.[39]. Because of its very high abundance in Ad5BM5-R2-infected 293 cells, the recombinant R2 can be readily purified to >95%. By contrast, the lower abundance of R2 in Bac-R2-infected Sf9 cells (~2% of TCP) resulted in a degree of purity of only 36%. The R2 protein, purified from Ad5BM1-R2-infected 293 cells, yielded an intermediate degree of purity, approximately 52%. The R2 molecular activity estimated from R2 content and reductase assays performed with excess of HSV-2 R1 was similar with either expression vectors, and essentially indistinguishable from

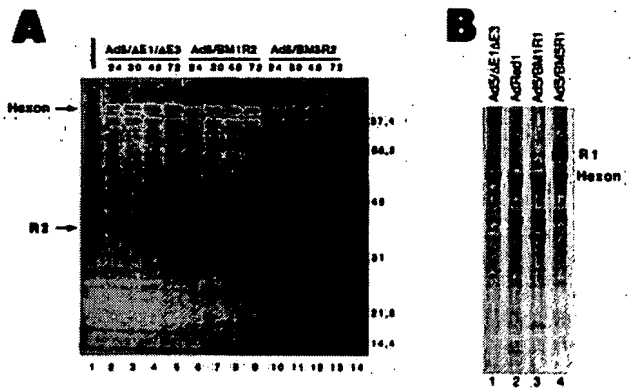

FIGURE 2. Expression of HSV-2 R2 and HSV-2 R1 in 293 cells infected with Ad5 recombinants. (A) HSV-R2. Coomassie blue stained gel of total proteins produced in 293 cells uninfected (lane 1), infected with Ad5/ΔE1ΔE3 (lanes 2–5), Ad5BM1-R2 (lanes 6–9), and Ad5BM5-R2 (lanes 10–13). Each lane was loaded with equal amounts of protein from total cell extracts. The position of hexon and R2 proteins are indicated on the left. Molecular weight markers are shown on the right. (B) HSV-R1. Coomassie blue stained gel loaded with equal amounts of total proteins produced in 293 cells infected for 48 h with Ad5/ΔE1ΔE3 (lane 1), AdRed-1 (lane, 2), Ad5BM1-R1 (lanes 3), and Ad5BM5-R1 (lanes 4). The position of R1 and hexon proteins are indicated on the right.

TABLE 1. R1 content and activity in extracts of cells infected with different recombinant viruses.

Recombinant virus	Time-post infection (h)	Total extract	Crude Extract		S_{100} extract	
		Content[a]	Content	Activity[b]	Content	Activity
Ad5BM1-R1	48	5.0	3.8	1.1	N.D.[c]	N.D.
Ad5BM5-R1	18	8.9	N.D.	0.7	1.5	0.5
	24	15.8	N.D.	2.4	5.0	2.5
	48	17.2	14.5	3.3	6.0	3.0
	72	21.2	N.D.	3.0	5.5	2.7
Bac-R1	48	17.0	N.D.	0.5	0.8	0.4
	72	N.D.	N.D.	0.25	N.D.	N.D.

[a]Determined by scanning of Coomassie blue stained gels using pure Ad5BM-R1 as standard and expressed in % of the total amount of protein loaded on the gel. [b]R1 specific activity determined using R2 in excess and expressed in U/mg of protein. [c]N.D., not determined.

the specific activity of authentic R2 produced in HSV-2-infected BHK cells as previously reported[22].

The expression of the recombinant protein in Bac-R1-infected Sf9 cells was far better than in Bac-R2-infected cells. Indeed, the level of recombinant R1 in total cell extracts, at the peak of accumulation, between 40–48 hours post-infection, was very close to the level found in Ad5BM5-R1-infected 293 cells, as evaluated by densitometric analysis of Coomassie blue stained gels (Table 1) or by immunoblotting (Fig. 4A). However, as can be seen in Figure 4A, degradation products detected with a polyclonal antiserum directed against the R1 protein, were much more abundant in total cell extracts with Bac-R1 than with Ad5BM5-R1. Using R2 in excess, R1 activity was measured in crude extracts of Bac-R1-infected Sf9 cells and of Ad5BM5-R1- or Ad5BM1-R1-infected 293 cells harvested 48 hours post-infection (Table 1). Unexpectedly, even if Bac-R1 and Ad5BM5-R1 expressed the recombinant protein at similar level, the R1 produced by the BEVS exhibited a 6-fold lower activity. When the R1 concentration was evaluated in these crude extracts, it was observed that a large proportion of the Bac-R1 protein had been spin out by the 12,000g centrifugation used to eliminate cellular debris. As this result suggested that the recombinant R1 was present mainly in an aggregated form, a 100,000g centrifugation was performed on the crude extracts to better evaluate the amount of soluble protein produced by both expression systems. Measurements of R1 concentration in S100 revealed that the proportion of soluble R1 was 7-fold higher in the 48 hour extract of Ad5BM5-R1 (6% compared to 0.8% for Bac-R1). The lower proportion of soluble R1 protein in the Bac-R1 extract is therefore the main cause of the lower R1 activity in this extract. Hence, by contrast with recombinant R2 which was fully soluble, it appears that in infected cells a significant fraction of R1 protein was aggregating presumably as a result of misfolding.

Since with the BEVS the amount of active R1 was not better at shorter or longer times post-infection, and since numerous attempts to denature and renature the Bac-recombinant protein were unsuccessful, we considered more advantageous, for the mass production of the protein, to seek to improve the yield of soluble R1 in 293 infected cells. Measurements of the R1 activity at different times post-infection with Ad5BM5-R1 showed that the activity increased in parallel with the accumulation of the protein (Fig. 4B), indicating that the percentage of soluble R1 was relatively constant (Table 1). After 60 hours, a decrease of activity presumably related to a loss in cell viability was often observed. This result indicated that the conditions of infection needed to be optimized in order to obtain maximal amount of active protein. In this regard, the two most important parameters were to infect the cells with a multiplicity of infection of at least 20 pfu/cell and to harvest them while the viability was still very high, usually before 48 hours post-infection. Hence, the amount of soluble R1 being consistently 7–8% of TCP, 15 mg of R1 could be purified per liter of 2×10^9 infected cells (to be published elsewhere). Unfortunately, coinfection of 293 cells with the Ad5BM5 recombinants for the two subunits, a strategy which was successful to diminish aggregation of some heterodimeric protein, did not significantly increase the yield of soluble R1.

Discussion

The application of Ad vectors for high level expression of recombinant proteins in animal cells shares the same niche as the very popular BEVS. Although developed after Ad vectors, BEVS soon emerged as a very powerful and versatile tool for the expression of a wide variety of heterologous proteins. This stems from the relatively lower complexity of gene regulation in the expression of some key Bac genes, namely polyhedrin

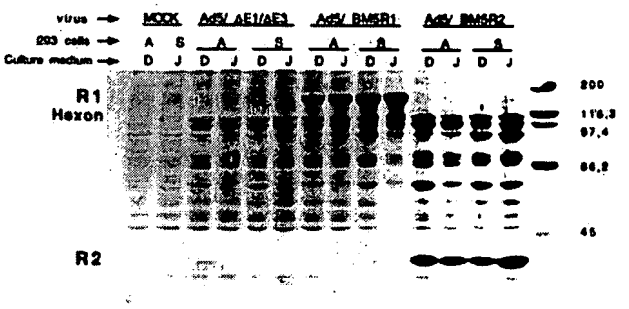

FIGURE 3. Scale-up of the Ad expression system. Coomassie blue stained gel of total proteins produced either in 293A (anchorage-dependent) or 293S (suspension) cells infected with Ad5/ΔE1ΔE3, Ad5BM5-R1, and Ad5BM5-R2 in DMEM+10% FBS and Joklik+5% CS in monolayer cultures. The position of R1, hexon, and R2 proteins are indicated on the left. Molecular weight markers are shown on the right.

and p10, which in turn allowed for the rapid construction of efficient transfer vectors. In addition to its high level of expression (up to 25% of TCP), this system offers the advantage of proven large-scale production capacity. However, careful characterization of numerous recombinant proteins has pointed to some problems in post-translational modifications in insect cells, such as impaired glycosylation, incomplete proteolytic cleavage of polyprotein precursors, and hypophosphorylation (see ref. 36 and references therein). These limitations restricts the utilization of BEVS for the production of numerous complex mammalian proteins, whenever authentic post-translational modifications are required for specific applications. In that respect, Ad vectors are better suited for the expression of mammalian proteins, particularly but not exclusively those of human origin. However, due to the complexity in the regulation of gene expression in Ad, the development of their full potential as very high level expression vectors lagged behind BEVS.

By including both the Ad MLP downstream activating sequences (R1, R2 and R3) and the BKV enhancer in our previous transfer vector pAdBM1 (ref. 22), the production of HSV-2 R1 and R2 proteins was boosted 3-fold to reach unprecedented levels of recombinant protein (15–20% of TCP) in human 293 cells. We have not yet precisely established the relative contribution of these two elements on the stimulation of transcription of the ectopic MLP in our new transfer vector pAdBM5. Nonetheless, preliminary characterization of expression cassettes, containing either of these *cis*-acting sequences separately, indicated that either elements are able to stimulate the expression of recombinant HSV-2 R2 with similar efficiency although not quite as much as the combination of both (data not shown). By including similar MLP downstream activating sequences in their transfer vectors, Mason et al.[26] have shown that the expression of the HBsAg could be stimulated more than 20-fold in Ad4 and Ad7 recombinants. Although this stimulation is more dramatic than the 3-fold increase we have observed, it should be noted that their 20-fold stimulation was obtained over an Ad construct expressing a relatively low level of recombinant protein and that even with their improved vector the level of HBsAg was lower than the level of the hexon. Moreover, when they cloned the MLP downstream activating sequences in their vectors, they also incorporated a splicing signal in a vector that did not contain any intron in the expression cassette. Given the importance of introns, which have been shown to stimulate gene expression from 2- to 500-fold depending on the gene and on the intron sequences[40], the 20-fold stimulation they observed could be accounted for, at least in part, by the influence of the intron they included[26].

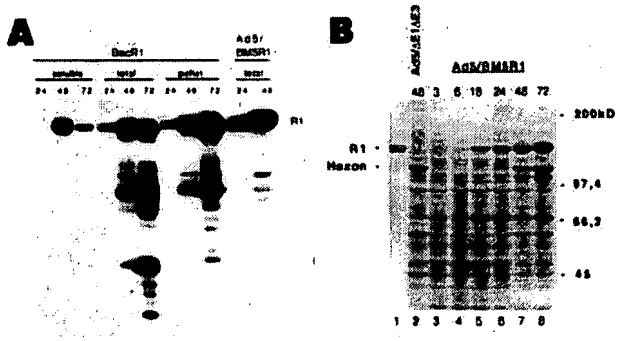

FIGURE 4. Analysis of amount of R1 in total and fractionated extracts of infected cells. (A) Western blot of Bac-R1-infected Sf9 cells and Ad5BM5-R1-infected 293 cells extracts at various time post-infection as indicated on the top. Total, soluble and pellet fractions were prepared as described in the Experimental Protocol. The position of R1 protein is indicated on the right. (B) Coomassie blue stained gel of total proteins produced in 293 cells infected with Ad5/ΔE1ΔE3 48 h post-infection (lanes 2), and Ad5BM5-R1 at 3, 6, 18, 24, 48, and 72 h post-infection (lanes 3–8). Each lane was loaded with 50 μg of protein from total cell extracts except lane 1 which was loaded with 4 μg of >95% purified R1. The position of hexon and R1 proteins are indicated on the left. Molecular weight markers are shown on the right.

In 293 cells infected with Ad recombinants generated with the pAdBM5 transfer vector, both HSV-2 R1 and R2 ribonucleotide reductase subunits represent the most abundant polypeptides, accounting for as much as 20% of TCP (Fig. 3). This is not unique to the HSV-2 R1 and R2 genes since other viral gene such as the EBV BARF-1 (P. Jolicoeur, B. Massie, and T. Ooka; unpublished) as well as some cellular genes including PTP1C[35,41], and human ribonucleotide reductase R1 and R2 subunits (N. Pavloff, F. Forghani, Y. Langelier, and B. Massie; unpublished) have been expressed at similar high levels with this vector. Furthermore, some results suggest that the actual level of expression obtained with the pAdBM5 derived recombinant is probably very close to the upper limit of the system. Indeed, the reduction in the level of hexon indicated that the very high level of production of the recombinant protein probably takes place at the expense of the Ad abundant late proteins. The observation that the titers of the improved recombinant viruses were significantly reduced is also consistent with this interpretation. Fortunately, the titer reduction did not affect the large-scale production of recombinant proteins with the Ad expression system. Thus, in spinner cultures of 293S infected with Ad5BM5-R2 and Ad5BM5-R1, 45 mg of R2 and 52 mg of R1 were produced per 1×10^9 cells. These yields fall within the range of what has been reported for the best Bac recombinants when the recombinant protein was precisely quantitated: 30 mg for SV40 LT antigen[42], 31 mg for a mammalian protein kinase[43], 45 mg for HBsAg[44], 50 mg for BTV VP6[45], and 90 mg for CAT[46].

When compared to the widely utilized BEVS, the performance of this improved Ad vector was found to be significantly better for the production and purification of active HSV-2 R1 and R2 subunits. The HSV-2 R1 subunit was produced in roughly equivalent amounts with either system, but its specific activity in soluble crude extracts was at least 5-fold higher in Ad5BM5-R1-infected 293 cells, presumably due to a better folding of the R1 protein in 293 cells. In Bac-R1-infected Sf9 cells, the improper folding of R1 was characterized both by its aggregation and degradation. The appropriate folding, assembly, and localization of newly synthesized proteins is dependent upon a group of proteins known as molecular chaperones (reviewed in refs. 47–49). Although little is known about the specific chaperone activity in Ad-infected 293 cells or Bac-infected Sf9 cells, it can be argued that the higher proportion of properly folded active R1 in 293 cells is likely the result of a better chaperone activity in Ad-infected 293 cells. To our knowledge, the aggregation of recombinant R1 in Ad-infected 293 cells is the first reported case of aggregation of overexpressed proteins with Ad vectors. Interestingly, it does not seem to be solely related to the magnitude of overexpression of recombinant R1 since the proportion of soluble protein remains constant whether it is expressed at a low level at the beginning of the late phase (18 hours) or at a higher level later on. If one assumes that the chaperone machinery is probably saturated in Ad-infected 293 cells, this result suggests that the recombinant R1 may be competing with the other Ad late proteins for the chaperone proteins and that the increase in properly folded R1 is made possible by the concomitant decrease in the amount of Ad late proteins. In other respects, aggregation of highly expressed recombinant proteins produced with BEVS has been reported. This was the case for the human 5-lipoxygenase which was found to be more than 50% insoluble[50], the catalytic subunit of protein phosphatase I which was expressed at a level of 20–25% of total protein but had less than 5% of the recombinant protein in a soluble form[51], and the glucocorticoid receptor[52] or the protein kinase C-δ[53] for which only less than 1% of the synthesized protein was active. As well, proteolytic degradation of recombinant protein in Sf9 cells has been observed previously with a number of genes, including the human 5-lipoxygenase[50] and BHV gIV[54]. The utilization of late promoter, such as the basic protein promoter, in place of the strong polyhedrin promoter, has been reported to improve the fraction of soluble protein from less than 1% to more than 10% in the case of the protein kinase C-δ although the expresion of total protein kinase C-δ was reduced by about 4-fold[55]. Thus, changing the temporal expression of recombinant proteins using weaker promoters can improve the quality of some complex proteins in insect cells[56].

We have also expressed both R1 and R2 in *E. coli* using the T7 expression vectors. The fraction of active soluble R1 in *E. coli* barely represents 1% of TCP and because of its lower abundance is much more difficult to purify than the R1 produced in Ad-infected 293 cells with a yield of only 0.1 mg of purified R1 per liter (to be published elsewhere). By contrast, the R2 produced in *E. coli* with the pET-R2 vector is twice as abundant as in Ad5BM5-R2-infected 293 cells (>30% of TCP) and as such even easier to purify. However, the R2 purified from *E. coli* has a 2.5-fold lower specific activity than the one purified from eukaryotic cells due to its lower content in tyrosyl radical which is essential for its activity[34].

Thus, given the ability to easily generate large volumes of Ad-infected 293S cells, and due to their higher relative abundance in the soluble fraction of the crude extract, both recombinant R1 and R2 were easy to produce and purify with the Ad expression system, and as such, it represented the best source to obtain large quantities of pure HSV-2 R1 and R2 necessary to study the structure of the HSV ribonucleotide reductase, an important target for anti viral chemotherapy.

Experimental Protocol

Cells and viruses. The 293A (adherent) cells are derived from human kidney fibroblasts transformed with Ad5 DNA and express the E1A and E1B proteins constitutively[57]. They were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories), supplemented with 10% fetal bovine serum (FBS, Gibco) glutamine and antibiotics. The 293S (suspension) cells, derived from the 293A cells adapted to grow in suspension, were kindly provided by Michael Matthew (Cold Spring Harbor) and grown as spinner cultures in Joklik's modified medium (Sigma) supplemented with 2.5g/l of glucose, 2.5g/l of hepes, 5% calf serum (CS) and antibiotics. The human Ad mutant Ad5/ΔE1ΔE3

(ref. 7), used as the parent virus in all our viral constructs, and the Ad recombinants were propagated by infecting monolayer 293 cells as described previously[18]. AdRed-1, a helper-free Ad recombinant expressing the HSV-2 R1 subunit[15], was kindly provided by Sylvia Bacchetti. Large-scale production of Ad stocks was done by infecting exponentially growing 293S cells (0.5×10^6 cells/ml) at a moi of 10–20 pfu/cell and harvesting the infected cells at 72 h post-infection. The cell pellet was resuspended in fresh Joklik's modified medium at a cell density of 1×10^8 cells/ml and virions were released by three to six cycles of freezing and thawing. Adenovirus titers were determined by plaque assay on 293A cells. *Spodotera frugiperda* (Sf9) cells were obtained from ATCC and grown as spinner cultures in TNMFH medium supplemented with 10% FBS and 0.1% Pluronic F-68 as described[37]. *Autographa californica* nuclear polyhedrosis virus (AcNPV), a baculovirus used to construct the Bac recombinants was kindly provided by Max Summers and propagated by infecting suspension Sf9 cultures as described previously[37]. Baculovirus titers were determined by plaque assay on monolayer cultures of Sf9 as detailed in Summers and Smith[37].

Expression vector constructions. All recombinant DNA molecules were constructed by standard cloning and site-directed mutagenesis procedures and propagated in *E. coli* DH5. The transfer vector pAdBM5 was derived from pAdBM1 (ref. 22) by sequentially subcloning three different synthetic oligonucleotides with compatible Bgl II ends as follows. Firstly, a double-stranded 73-mer oligonucleotide containing one copy of the 68 bp repeat of the BKV enhancer (Dunlop isolate[38]) was cloned at the Bgl II at map position 380 on pAdBM1; secondly, a double-stranded 198-mer oligonucleotide containing one copy of the entire BKV enhancer (Dunlop isolate[38]) was cloned at the Bgl II at map position 2590 on pAdBM1; and finally a double-stranded 108-mer oligonucleotide containing the downstream late-phase-specific activating sequences of the Ad MLP, mapping between +30 and +130 relative to the transcription start site[24], was cloned at the Bgl II at map position 2010 on pAdBM1 (Fig. 1). pAdBM1-R1 was constructed by cloning the BamH I fragment of the plasmid A392-B (kindly provided by Sylvia Bacchetti) encoding the HSV-2 R1 subunit into the Bam HI site of pAdBM1. Before this insertion, the initial plasmid A392-B had been modified to replace the 5'-TCCATGG-3' motif at the R1 initiator codon with an optimal Kozak's consensus sequences (underlined) for efficient translation[39], by cloning a double-stranded 23-mer oligonucleotide 5'-CCATGGATCC(GCC)$_3$ATGG-3' at the Bal I site. Insertion of the synthetic DNA restored the initiator ATG in frame and created a new Bam HI site in adjacent upstream sequences. pAdBM5-R1 was constructed by cloning the Bam HI fragment of the modified A392-B plasmid at the Bam HI site of pAdBM5. pAdBM5-R2 was constructed by cloning the Bgl II-Bcl I fragment encoding the HSV-2 R2 subunit from pSVRR.^{HSV} at the Bam HI site of pAdBM5 as previously described[22]. For expression in Bac, the same Bam HI fragment encoding the HSV-2 R1 subunit that was cloned in pAdBM1 was blunt-end ligated into the Nhe I cloning site of the BlueBac transfer vector pJVETL[40] (kindly provided by Chris Richardson) to produce pJVETL-R1. The same Bgl II-Bcl I fragment encoding the HSV-2 R2 that was cloned in Ad vectors, was also cloned at the Bam HI site of the transfer vector pAc373I to produce pAc373I-R2. pAc373I is a derivative of pAc373 (ref. 57) in which the missing 8 nucleotides from the polyhedrin leader were restored while deleting 10 nucleotides derived from the polylinker used to introduce the Bam HI cloning site in pAc373.

Construction of HSV-2 R1 and R2 recombinant viruses. The generation of Ad5BM1-R2 recombinant (formerly named Ad5BM1-RR$_2^{HSV}$) has been described previously[22]. The other Ad recombinants, Ad5BM1-R1, Ad5BM5-R1, and Ad5BM5-R2 were constructed with the appropriate plasmids essentially in the same way with minor modifications[24]. Briefly, the Ad transfer vectors pAdBM1-R1, pAdBM5-R1 and pAdBM5-R2 were linearized at the unique Cla I site and rescue into the genome of Ad5/ΔE1ΔE3 by *in vivo* homologous recombination between overlapping sequences on the transfer plasmid and the large right-end fragment of Ad5/ΔE1ΔE3 genome, upon cotransfection of 293A cells. Digestion of Ad5/ΔE1ΔE3 viral DNA with Cla I prior to transfection allowed for obtainment of recombinant Ad at a frequency of 5 to 50%. Seven to ten days post-transfection, 24 viral plaques were picked, amplified and tested either for HSV- R1 or R2 expression by Western blotting. For R1 detection, we used a polyclonal serum obtained from rabbits immunized by a first injection of partially purified insoluble R1 produced by Bac-R1 with complete Freund adjuvant, followed by a boost with purified soluble R1 produced by Ad5BM5-R1. This serum did not react against wild type Bac-infected Sf9 cells nor Ad-infected 293 cells. R2 was detected with a rabbit antiserum raised against a nonapeptide corresponding to the C-terminus of the R2 protein[41]. At least two positive independent plaque isolates were further purified by two consecutive rounds of plaque assays, and viral stocks were prepared as described above. The Bac-R2 recombinant was generated from pAc373I-R2 using the standard procedure detailed in Summers and Smith[37]. The Bac-R1 recombinant was generated from pJVETL-R1 following the same procedure improved by the visual screening of blue recombinant plaques as described by Vialard et al.[42].

Production of Ad recombinant proteins. For the time course analysis of recombinant protein synthesis or accumulation, Petri dishes of sub confluent 293A cells or exponentially growing 293S cells were infected at a density of 0.5×10^6 cells/ml with a viral inoculum corresponding to a moi of 25–50 pfu/cell. At different times post-infection, total protein extracts were prepared by lysing PBS washed cells with 2% SDS in 80 mM Tris-HCl pH 6.8 and 10% glycerol. Before protein analysis by SDS-PAGE and Western blotting performed as described previously[22], the extracts were passed several times through a syringe needle or sonicated to shear the DNA. After determination of protein concentration using the Bio-Rad *DC* (Detergent Compatible) colorimetric assay with BSA as standard, DTT was added to a concentration of 5% (V/V) and the samples were boiled for 5 min before loading on gel. Quantification of the % of recombinant R1 and R2 in total protein extracts was done by densitometric scanning of Coomassie blue stained gels with a Jandel Video Analysis System (Jandel Scientific) or by immunoblots using pure R1 or R2 proteins as standards. For assays of ribonucleotide reductase activity on the unpurified recombinant subunits, infected cells were washed with PBS and suspended in ice-cold buffer A (50 mM Hepes pH 7.6, 2 mM DTT). Crude protein extracts were obtained by a 2 min sonication followed by centrifugation at 12,000g for 10 min at 4°C as previously detailed[22]. The supernatants (S_{12}) were then centrifuged through Sephadex G-25 columns to remove molecules inhibitory for reductase activity. Protein concentration of the protein extracts prepared without detergent was determined with the Bio-Rad (Bradford) protein assay using BSA as standard. For the mass production of the recombinant subunits, spinner cultures (3 to 6 L) of 293S cells at a density of $1.5\text{-}2.5\times10^6$ cells/mL were pelleted, resuspended at 1×10^7 cells/ml, incubated with a viral inoculum corresponding to a moi of 25–50 pfu/cell for 2 h, diluted to 2×10^6 cells/ml with fresh Joklik's medium, and incubated at 37°C. The medium was replaced 24 h later and the infected cells were harvested usually 48 h post-infection. S_{12} extracts were prepared from PBS washed cells as described above.

Production of Bac recombinant proteins. Bac infections of Sf9 cells were done under optimal conditions as detailed previously[37]. Briefly, Sf9 cells were grown in 4L bioreactors (Celligen™) and infected with recombinant Bac with a moi of 10 pfu/cell when the culture had reached typically $1.5\text{-}2\times10^6$ cells/ml. Cells were harvested 48 to 72 h post-infection and protein extracts (total or crude) were prepared as described above for Ad-infected 293S cells.

Purification of HSV-2 R2 and R1 recombinant proteins. The purification of the R2 subunit was accomplished following the two steps method of Lankinen et al.[39], with some modifications that will be described elsewhere[34]. Briefly, the S_{12} extract was successively precipitated with 1.0% streptomycin sulfate and ammonium sulfate (25% saturation). The ammonium sulfate precipitate was dialyzed twice against buffer B (20 mM BisTris-HCl pH 5.8, 10% glycerol), and the R2 protein was further purified by anion exchange chromatography on a FPLC MonoQ hr10/10 column (Pharmacia). The concentration of the R2 protein was determined by light absorbency using an extinction molar coefficient ($\epsilon_{280\text{-}310}$) of 52,000 M^{-1} cm^{-1} (ref. 63). The protein purity was evaluated to be superior to 95% by laser densitometric scanning of a lane containing 10 ug of protein on a Coomassie blue stained gel. The purification of the R1 subunit was based on its affinity for the dodecapeptide STSYAGAVVNDL corresponding to the C terminus of the HSV-2 R2 subunit. The procedure modified from the one originally developed for R1 expressed at low level in HSV infected cells[32] will be detailed elsewhere (Champoux et al., in preparation). Briefly, crude cytoplasmic extracts were prepared by Dounce homogenization followed by a 12,000g centrifugation. The S_{12} were further clarified by centrifugation at 100,000g for 1 h at 4°C. Between 100 to 150 mg of these S_{100} proteins were loaded on a 40 ml column of Affiprep coupled peptide. The R1 protein was eluted from the column with a yield of ~50% using the peptide Ac-YAGAIVNDL. The protein purity was evaluated to be superior to 90% by laser densitometric scanning of a lane containing 5 µg of protein on a Coomassie blue stained gel.

Ribonucleotide reductase assay. R2 specific activity was determined by adding to limiting amounts of R2 excess amounts of S_{100}-R1 (specific activity: 3 U/mg). In some cases, it was verified that similar R2 activity values were obtained with purified R1(specific activity: 55 U/mg). R1 specific activity was determined by adding to limiting amounts of R1 excess amounts of R2 purified from *E. coli* expressing the pET-R2 vector (specific activity: 60 U/mg[34]). Ribonucleotide reductase activity was assayed by monitoring the reduction of [^{3}H]-CDP[64]. The standard reaction mixture contained 50 mM Hepes pH 7.8, 4 mM NaF, 50 µM CDP, and 0.25 µCi of [^{3}H]-CDP in a final volume of 60 µl. One unit of ribonucleotide reductase was defined as the amount of enzyme generating 1 nmol of dCDP/min.

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Exhibit 4

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Adenovirus Inhibition of Cell Translation Facilitates Release of Virus Particles and Enhances Degradation of the Cytokeratin Network

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Infection of animal cells by a number of viruses generally results in an array of metabolic defects, including inhibition of host DNA, RNA, and protein synthesis, and morphological alterations known as cytopathic effects. For adenovirus infection there is a profound loss of cell structural integrity and a marked inhibition of host protein synthesis, the latter generally assumed necessary to enhance virus production. We examined the purpose of viral inhibition of cell translation and found that it was related in part to cytopathic wasting of infected cells. We show that viral shutoff of host translation promotes destruction of the intermediate filament network, particularly cytokeratins which are proteolysed at keratins K7 and K18 by the adenovirus late-acting L3 23-kDa proteinase. We found that if adenovirus is prevented from inhibiting cell translation, the intermediate filament network remains relatively intact, keratin proteins are still synthesized, and cells possess an almost normal morphological appearance and lyse poorly, reducing the release of nascent virus particles by several hundredfold. Remarkably, in tissue culture cells the accumulation of late viral structural proteins is only marginally reduced if host translation shutoff does not occur. Thus, a surprising major function for adenovirus inhibition of cellular protein synthesis is to enhance impairment of cellular structural integrity, facilitating cell lysis and release of progeny adenovirus particles.

Many viruses mediate cytopathic effects (CPE) in host cells through a variety of poorly understood molecular mechanisms, in some cases culminating in death of the cell (for a general review, see reference 54). On the other hand, some viruses cause persistent infections and replicate for extended periods of time without producing noticeable CPE. Cells productively infected with human adenovirus (Ad) classically demonstrate a significant CPE which becomes progressively more obvious during the late phase of the viral replication cycle. The late phase of infection is typified by replication of Ad genomic DNA, activation of the viral major late transcription unit which encodes late viral polypeptides, suppression of host and early viral mRNA translation, and preferential translation of late viral mRNAs (reviewed in reference 46). Attendant CPE is manifested by dramatic morphological alterations of infected cells, including rounding, clumping, and detachment from the monolayer (reviewed in reference 20), cessation of cellular DNA and protein synthesis (27), dissolution of the cytoskeleton (14, 55, 57, 59, 65), and eventually cell lysis.

The causes of Ad pathogenic effects are not well understood, but appear to involve a variety of different factors depending on whether in vivo infection of animals or in vitro infection of cultured cells is studied. For instance, there is limited evidence that early viral gene expression may be directly cytotoxic in some cultured cells (11, 17, 66a). There is considerably more evidence, however, that during natural infection early Ad gene expression indirectly causes extensive pathological effects by provoking a cell-mediated immune response directed against early viral proteins presented on the cell surface or by inducing elaboration of cytokines from infected cells (28, 43).

Immune attack of infected cells obviously cannot be responsible for the severe CPE typically evident at late times during Ad infection of cultured cells and may be only one factor that contributes to CPE during natural infection. Ad-mediated CPE could include an extensive collapse of cellular intermediate filament networks, which is mediated by several viral gene products. The vimentin network degenerates about the perinucleus at early times after Ad infection (14, 65), apparently by activation of a cellular protease triggered solely by viral particles (6). Collapse of vimentin and lamin networks is also mediated by the Ad early E1B 19-kDa protein in transfected cells (56, 57). In addition, it has recently been shown that during the late phase of Ad infection cytokeratins K18 and K7 are degraded by the viral late acting L3 23-kDa proteinase, leading to collapse of the keratin network (10). The disruption of cellular intermediate filament networks would be expected to contribute to CPE by impairing the structural integrity of the cell.

Few studies have systematically investigated the basis for severe pathogenic effects that occur during the late phase of Ad replication or questioned the purpose of Ad inhibition of host protein synthesis. Several inconclusive early reports studied the effect of adding high concentrations of late Ad structural polypeptides to cells in culture or to cell extracts (reviewed in reference 26). A recent study demonstrated that infection of cotton rat lungs by a replication-defective Ad virus that expressed early but not late viral genes caused extensive pathology but less than infection with wild-type (wt) Ad virus (28). Another study found that during infection of primary cultures of kerotinocytes CPE occurred only if Ad progressed into its late phase of replication (3). We therefore asked whether key events of late Ad replication are directly related to CPE, such as virus-mediated shutoff of cellular protein synthesis or the potentially toxic accumulation of late Ad structural polypeptides. Several experimental approaches were used, including infection of cell lines that are resistant to Ad-controlled translation inhibition, the ability to prevent late Ad shutoff of host protein synthesis by treatment of cells with

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2-aminopurine (2AP), and infection with a temperature-sensitive (*ts*) mutant in the viral late-acting L3 proteinase. We describe a surprising major function for late Ad inhibition of cellular protein synthesis, which is to prevent synthesis of new keratins and restoration of the cytokeratin network that is degraded during late infection. We found that viral proteolysis of keratin filaments coupled to inhibition of host translation prevents the cell from maintaining its structural integrity, which is crucial for the efficient release of infectious Ad particles.

MATERIALS AND METHODS

Viruses, cells, and plasmids. Ad 300 is a wt strain 5 isolate (H5*wt*300) originally purified by H. Ginsberg. Ad *dl*309 is a phenotypically wt strain with altered restriction enzyme sites (34). Ad2 *ts*1 was provided by D. Ornelles (Wake Forest University). 293 cells are a human embryonic kidney cell line that express the E1 region of Ad5 (31). 293 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. Virus stocks were grown and titers were determined on 293 cells. RD (human rhabdosarcoma) cells were obtained from the American Type Culture Collection and grown in DMEM containing 15% fetal bovine serum. 2AP was prepared and used as previously described (32). Infections of cells with viruses were typically carried out for 1 h with 50 PFU per cell unless otherwise noted.

Labeling of cells and analysis of polypeptides. Cells were labeled with [^{35}S]methionine for 1 to 2 h by using 50 μCi of *trans*[^{35}S]methionine (ICN) per ml in DMEM lacking methionine and supplemented with 2% calf serum. Cell extracts were prepared by sonication of washed cells in 10 mM KCl–10 mM Tris (pH 7.4)–1 mM EDTA at 4°C and cleared of debris by centrifugation at 10,000 × *g*; equal amounts of protein were analyzed in sodium dodecyl sulfate (SDS)-polyacrylamide gels. Gels were fluorographed and quantitated by densitometry. Immunoprecipitation analysis was performed with equal amounts of protein in cell extracts, using specific antisera and protein A-Sepharose beads (Sigma Chemical Co.). Immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and fluorography. Antisera consisted of a polyclonal antibody directed against the E1B 19-kDa protein (provided by E. White, Rutgers University; 59) or a monoclonal antibody directed against hsp/hsc 70 proteins (J. Thomas, New York University).

Indirect immunofluorescence staining and photography of cells. Cells were grown on coverslips, fixed with paraformaldehyde, and permeabilized with Triton X-100 as described previously (61). Mouse monoclonal antibodies directed against vimentin were purchased from Boehringer Mannheim Biochemicals. A mouse monoclonal antibody specific for keratins K18 (KS-B17.2) and K1, 5 to 8, 10, 11, and 18 (no. 8.13) were from Sigma Chemical Co. Fluorescein isothiocyanate-conjugated rabbit anti-mouse antibodies were purchased from Sigma. Cells were photographed under visible or ultraviolet light using a Zeiss photomicroscope.

Extraction and biochemical analysis of insoluble keratin filament proteins. Intermediate filaments were extracted by the method of Wu et al. (62) as modified by Vassar et al. (53). Briefly, cells were scraped from plates, washed twice in cold phosphate-buffered saline, resuspended in 20 mM Tris-HCl (pH 7.4)–0.6 M KCl–1% Triton X-100, and then lysed by sonication at 4°C. An insoluble fraction was derived by centrifugation for 20 min at 10,000 × *g*; the pellet was resuspended in the same buffer and centrifuged again. The insoluble pellet was then resuspended in 8 M urea–10% β-mercaptoethanol, protein levels were determined using Bio-Rad reagent, and equal amounts were subjected to SDS-polyacrylamide gel electrophoresis.

Western immunoblot analysis. Equal amounts of protein samples were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, blocked, and probed with keratin K-18-specific immunoglobulin G monoclonal antibody (KS-B17.2; Sigma) or one that recognizes keratins K1, 5 to 8, 10, 11, and 18 (no. 8.13, Sigma). Antibodies were detected by binding to ^{125}I-protein A (New England Nuclear) and quantitated by densitometry of autoradiograms.

RESULTS

Absence of CPE in late Ad-infected cell lines resistant to translation inhibition. Previous studies found that several cell lines are capable of supporting productive Ad replication without permitting viral suppression of host protein synthesis at late times after infection (33, 39). One such line, a rhabdosarcoma (RD) cell line, maintains normal levels of host and somewhat reduced levels (~three- to fourfold lower) of late viral protein synthesis (39), which correlated with resistance to Ad-mediated inactivation and dephosphorylation of cap binding protein (also called eIF-4E) (33). RD cells were therefore infected with wt Ad5 virus (Ad 300), and the level of protein synthesis was monitored by metabolic labeling with [^{35}S]methionine and SDS-polyacrylamide gel electrophoresis. Plates of cells were also observed and photographed until 3 to 4 days postinfection (p.i.), at which point cells detached from the monolayer.

Figure 1A demonstrates the inability of Ad to inhibit host protein synthesis in RD cells, which was readily apparent by the diffuse background of labeled cellular polypeptides and the equal levels of actin protein present in uninfected and late Ad-infected cells. As shown previously by several laboratories (33, 39), accumulation of late Ad structural polypeptides and viral particles is reduced by ~two- to fivefold in these cells. Remarkably, few if any morphological features associated with Ad-induced CPE were found in these cells (Fig. 1B), including cell rounding and swelling. Instead, centers of infection were observed to display slight distortion immediately preceeding cell detachment from the monolayer and occasionally cell lysis, leaving large areas devoid of cells by day 3 as shown. Identical results were also obtained in GM2767A cells (data not shown), another line resistant to late Ad translation inhibition (45), suggesting but not proving that CPE might correlate with inhibition of cellular translation. However, given the inability to directly compare CPE and viral translational control in similar cells that undergo shutoff, we instead chose a different approach to this problem.

2AP prevents Ad shutoff of cell protein synthesis and induction of CPE. Previous work showed that the drug 2AP could prevent the shutoff of cell translation during late Ad infection while maintaining high rates of viral protein synthesis and accumulation (32), by blocking virus-mediated underphosphorylation of translation factor eIF-4E (33). Late Ad mRNAs continue to translate because they possess a common 5′ noncoding region called the tripartite leader (16) which apparently recruits minute amounts of remaining active factor (51) through preferential binding to an unstructured 5′ end (66; reviewed in references 46 and 50). Since most late viral polypeptides are synthesized at near-normal levels in cells treated with 2AP (32), this agent can be used to determine whether CPE results from potential cytotoxic effects of a late Ad polypeptide, from inhibition of cellular protein synthesis, or from alteration of other cellular processes.

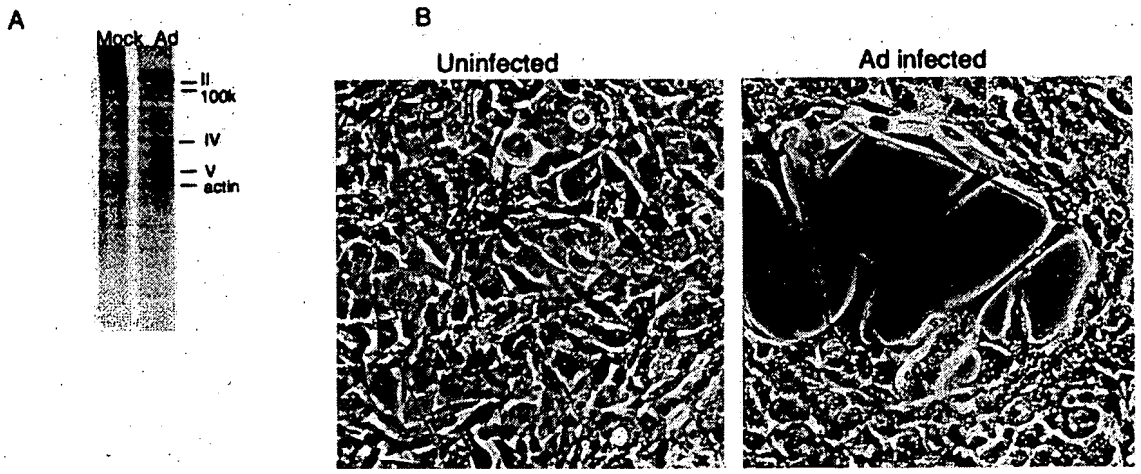


FIG. 1. Pattern of protein synthesis and changes in cellular morphology in uninfected and late Ad-infected RD cells. Cells were infected with 50 PFU of wt Ad per cell and analyzed 48 h later. (A) Cells were labeled with 50 μCi of [^{35}S]methionine per ml for 2 h, lysates were prepared, and equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis and fluorography. Late Ad polypeptides are indicated. (B) RD cells were photographed at 48 h p.i. A typical area in which infected cells have detached from the monolayer is shown.

293 cells were treated with 2AP for the duration of infection, starting shortly after the addition of virus, as previously described (32). Duplicate plates of cells were then labeled with [^{35}S]methionine, and extracts were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2) or photographed at various times after infection for evidence of CPE (see Fig. 4). The ability of 2AP to prevent Ad shutoff of cell protein synthesis while maintaining high rates of translation for late Ad mRNAs was apparent (Fig. 2). Levels of cellular polypeptide synthesis (e.g., actin and background bands) as well as of Ad mRNAs which lack the tripartite leader (e.g., protein IX) were all significantly elevated by treatment of cells with 2AP. Immunoprecipitation analysis of selected polypeptides from labeled extracts indicated that preferential translation of late Ad mRNAs suppressed synthesis by severalfold of several cellular and early viral proteins that could potentially protect against cytotoxicity (Fig. 3). These include the heat shock hsp/hsc 70 proteins (Fig. 3A), which maintain normal cell viability (reviewed in references 13 and 25), and the Ad early E1B 19-kDa protein (Fig. 3B), shown to block cytotoxicity and apoptosis associated with expression of E1A proteins and tumor necrosis factor alpha (30, 44, 58, 60). Western immunoblot analysis of both hsp/hsc 70 and E1B 19-kDa proteins showed a two- to threefold decrease in steady-state levels as well (data not shown).

Most striking, however, was the large reduction in CPE in Ad-infected 293 cells treated with 2AP (Fig. 4). 293 cells are exquisitely sensitive to late Ad CPE as shown and generally displayed severe morphological alterations such as swelling, detachment from the monolayer, and lysis by 24 to 48 h after infection. Infected cells treated with 2AP demonstrated only slight CPE at 24 and 48 h. As observed with infected RD cells, by 48 h after infection some 2AP-treated 293 cells began to detach from the monolayer but there was little evidence that large numbers of cells lysed. Uninfected cells treated with 2AP did not demonstrate evidence of drug toxicity until ~3 days after treatment (data not shown; 32). Similar effects of 2AP on Ad-induced CPE were also observed for infected KB and HeLa cells (data not shown). These results therefore indicated that accumulation of abundant amounts of late Ad polypeptides was not particularly cytotoxic and was not likely to account for CPE during the late phase of infection.

FIG. 2. 2AP prevents the shutoff of translation of cellular and early Ad mRNAs. Uninfected and wt Ad-infected 293 cells were labeled with [^{35}S]methionine at 24 h p.i., and duplicate plates were treated with 10 mM 2AP, added 1 to 2 h after infection as described previously (32). Equal amounts of protein from labeled extracts were analyzed by SDS-polyacrylamide gel electrophoresis and fluorographed. Ad late polypeptides correspond to proteins II, III, IV, V, pIV, pVIII, pVI, and IX.

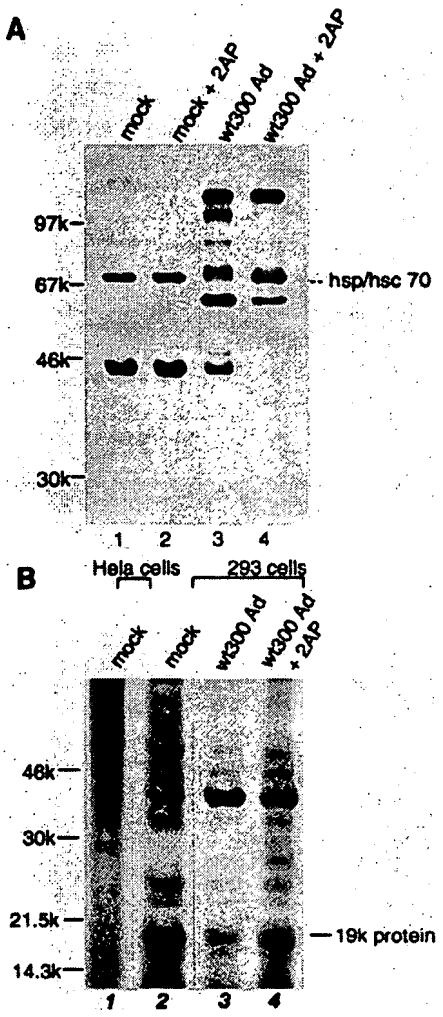


FIG. 3. Synthesis of hsp/hsc70 and E1B 19-kDa proteins is suppressed during the late phase of Ad infection. 293 cells were infected with wt Ad at 50 PFU per cell in the absence or presence of 2AP treatment. Uninfected (mock) and infected cells were labeled for 1 h with 50 μCi of [^{35}S]methionine per ml at 20 h p.i., lysates were prepared, and equal amounts of protein were used for immunoprecipitation with antibodies directed specifically against hsp/hsc 70 proteins (A) or the E1B 19-kDa protein. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Quantitation of bands was performed by densitometry. The positions of molecular size markers are indicated. A HeLa cell sample was included as a control in panel B because it is devoid of the E1B 19-kDa protein.

2AP is an inhibitor of several protein kinases (19), and it is therefore not surprising that treatment of cells results in a range of effects, including the ability to override cell cycle control checkpoints (2), inhibition of transcriptional activation by double-stranded RNA and interferon (52, 67), possible inhibition of the activity of the double-stranded RNA-activated inhibitor (DAI) kinase (35, 67), and rescue of poliovirus mutant 2A protease activity (40). It was therefore of concern that the ability of 2AP to prevent late Ad-induced CPE might be a general phenomenon, unrelated to prevention of Ad translation shutoff. This possibility was addressed by determining whether 2AP could prevent CPE caused by poliovirus and influenza virus, both of which shut off cellular protein synthesis. We found that 2AP was unable to prevent CPE caused by poliovirus and, in agreement with a previous report (40), did not prevent translation shutoff or greatly alter poliovirus replication. Viral translation and shutoff of host protein synthesis in influenza virus-infected cells was also not appreciably affected by 2AP treatment (data not shown). Thus, 2AP is not a general inhibitor of virus-mediated CPE and translation shutoff but rather acts with specificity in Ad-infected cells.

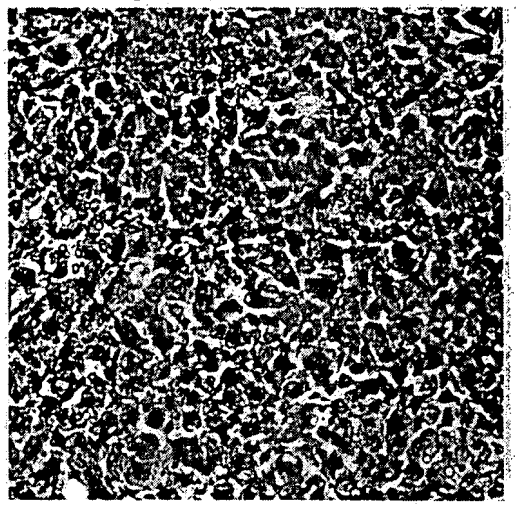
Disruption of vimentin filaments is not sufficient to cause Ad-associated CPE. Several Ad polypeptides have been shown to disrupt different components of the network of cellular intermediate filaments, either during early virus infection as shown for vimentin (14, 65) or in the late phase as shown for cytokeratins (10). We asked whether CPE results from viral disruption of different families of intermediate filaments by examining their organization in late Ad-infected 293 cells, with and without 2AP treatment (Fig. 5). Cells were grown on coverslips, infected with wt Ad, and then fixed and processed for indirect immunofluorescence by using an antibody directed against vimentin, which was shown previously to indicate the collapse of these filaments during infection (10, 56). As expected, uninfected cells possessed a highly organized network of vimentin filaments, which was found to be extensively disrupted during late Ad infection. Treatment of uninfected cells with 2AP for 48 h did not alter the normal organization of vimentin filaments. Surprisingly, infected cells treated with 2AP, which appeared morphologically similar to uninfected cells (Fig. 4), still showed extensive disruption of the vimentin network. The collapse of intermediate filaments in uninfected cells treated with 2AP was slightly less extensive than that in untreated infected cells, but still was quite significant. We can conclude that the disruption of vimentin filaments during late Ad infection was not sufficient to generate the CPE typically observed. The lack of correlation between collapse of the vimentin network and Ad-induced CPE is understandable, however. Many cells lack vimetin networks but synthesize them in culture, suggesting that the network may not be vital for the structural integrity of these cells (18).

Degeneration of the cytokeratin network requires shutoff of host translation and Ad L3 23-kDa proteinase activity. An intact cytokeratin network has been shown to be vital for the maintenance of normal cell structure, in that disruption of keratin filaments leads to cell lysis by only mild mechanical stresses (12, 53). The Ad L3 23-kDa proteinase, which is required at late times after infection for morphogenesis of viral capsid proteins (7, 64), has also been shown to cleave cytokeratin K18 and probably K7, thereby disrupting the keratin network during late infection (10). We therefore determined whether a primary function of host translation shutoff by Ad is to prevent repair of the proteolysed cytokeratin network, leading to extensive loss of cellular structural integrity and shape.

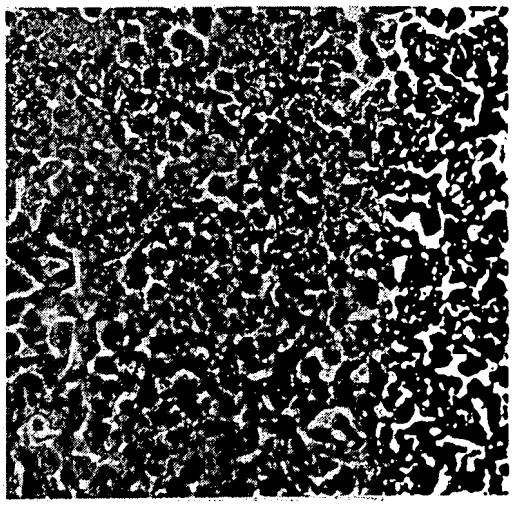
Recent work by Chen et al. (10) showed that during the late phase of Ad infection in HeLa cells, the L3 23-kDa proteinase cleaves the amino-terminal head domain of cytokeratin K18 and probably K7, resulting in disassembly of the intermediate filament network into cytoplasmic clumps. In our experiments, 293 kidney epithelial cells were used because they demonstrate a severe CPE and possess a cytokeratin network more related to bronchial epithelium, the natural host tissue for infection by Ad2 and Ad5 (22).

Cells were infected with wt Ad in the presence or absence of 2AP treatment and then fixed for indirect immunofluorescence by using an antibody directed specifically against keratin K18. Visual inspection of uninfected 293 cells demonstrated a

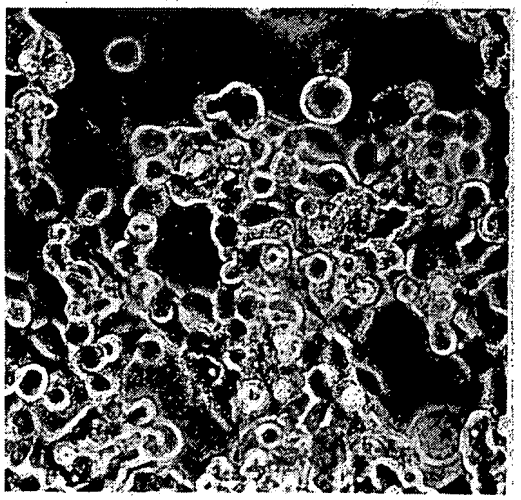
Uninfected



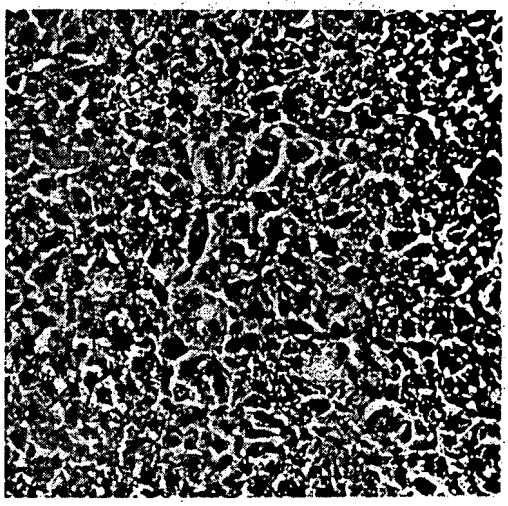
Uninfected + 2AP 48hrs



wt300 Ad infected 24hrs



wt300 Ad + 2AP 24hrs



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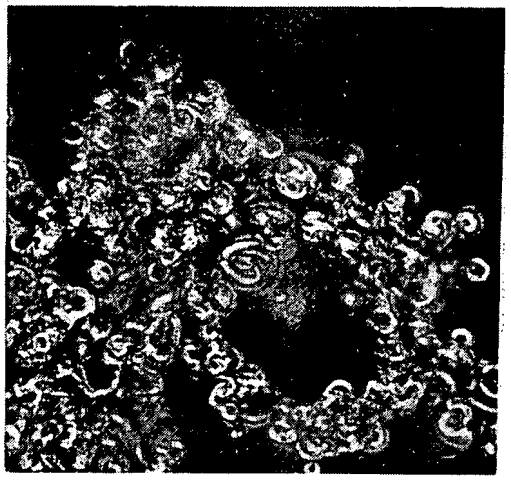

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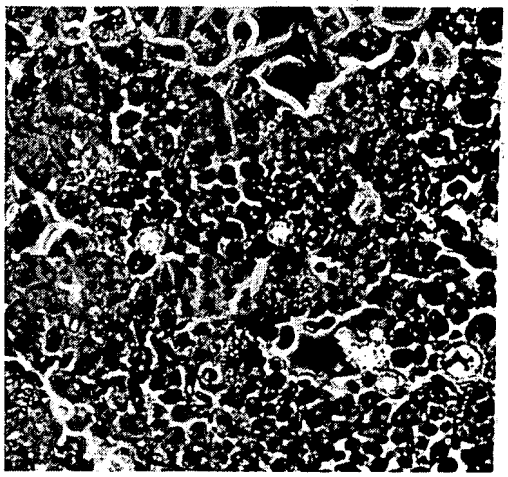

FIG. 4. Effect of 2AP on Ad-induced late CPE in 293 cells. Cells were infected at 50 PFU per cell with wt Ad, and 10 mM 2AP was added to duplicate plates 2 h p.i. for up to 48 h. Cells were photographed at 24 and 48 h. 2AP treatment of uninfected 293 cells had little detectable cytotoxicity at 48 h posttreatment as shown and largely prevented detectable CPE in wt Ad-infected cells observed as late as 48 h p.i.

normal pattern of ordered keratin cables (Fig. 6). 2AP treatment did not alter the normal appearance of keratin networks in uninfected cells. Cells infected with wt Ad for 24 h (late phase) displayed extensive disruption of keratin filaments, ranging from accumulation of large cytoplasmic aggregates to numerous spheroid globules. This pattern of disruption is typically observed after amino-terminal cleavage of keratin filaments in experimental systems (53), in epidermolysis bullosa simplex skin disorders (12), and in late Ad-infected HeLa cells (10). As expected, late Ad-infected cells also showed gross morphological changes typical of viral CPE. As shown earlier, cells infected with wt Ad but treated with 2AP possessed only slight evidence of CPE by light microscopic examination. Most striking was the pattern of the cytokeratin network in these cells. Only very limited amounts of disassembly (clumping and globules) could be detected in the presence of 2AP, despite

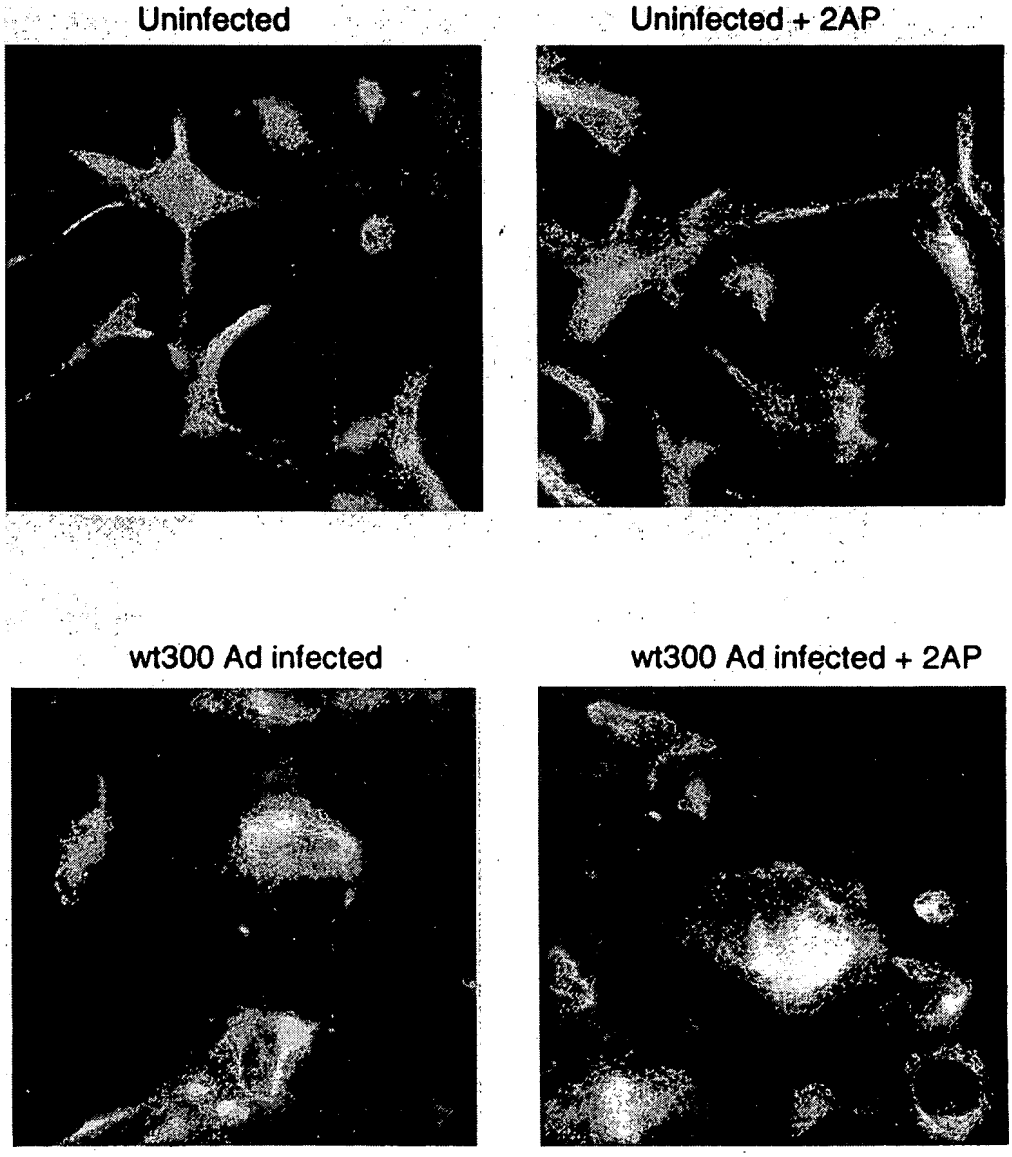

FIG. 5. Changes in vimentin filaments during late Ad infection. 293 cells grown on coverslips were infected with 50 PFU of wt Ad per cell in the absence or presence of 10 mM 2AP. At late times after infection when untreated infected cells showed extensive CPE, cells were fixed and processed for indirect immunofluorescence by using an antibody directed specifically against vimentin. Extensive disruption of vimentin filaments was observed during late infection, regardless of treatment with 2AP and the absence of CPE.

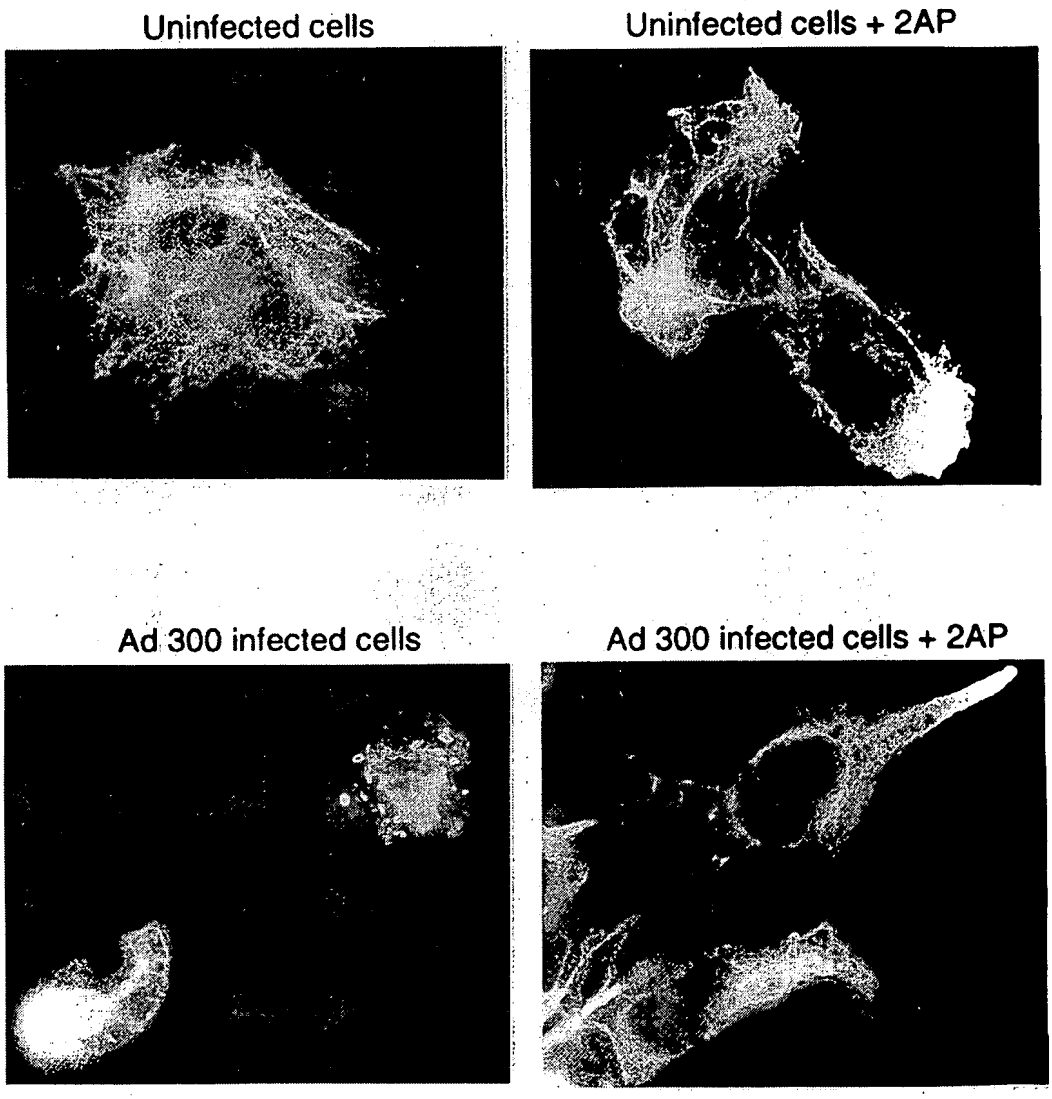

FIG. 6. The cytokeratin network is not extensively disrupted in late Ad-infected cells treated with 2AP. 293 cells grown on coverslips were infected at 50 PFU per cell with wt Ad in the absence or presence of 2AP. When untreated infected cells developed extensive CPE (~24 h p.i.), they were fixed and processed for indirect immunofluorescence by using an antibody directed specifically against keratin protein K18. Only slight disaggregation of cytokeratin filaments was observed in late Ad-infected cells treated with 2AP.

productive virus infection. Most networks were relatively intact, and only slight evidence was observed of intensely staining cytoplasmic clumps, typical of keratin proteolytic cleavage. However, most keratin cables were also not as well defined as those in uninfected cells, again indicative of some disruption.

The biochemical integrity of cytokeratins was next analyzed to determine whether blocking Ad translation shutoff also prevented accumulation of degraded keratin proteins. Insoluble keratin networks were extracted from uninfected and late Ad-infected cells in the absence or presence of 2AP treatment and resolved by SDS-polyacrylamide gel electrophoresis, and individual keratins were identified by immunoblot analysis, using specific antibodies (Fig. 7A). Blots probed for keratin K18 demonstrated roughly equal amounts of 45-kDa K18 protein in uninfected cells regardless of 2AP treatment. Cells infected with Ad for 24 h contained mostly the 41-kDa K18 cleavage product, indicating almost quantitative proteolysis of the amino-terminal head domain (10). It is also noteworthy that in late Ad-infected cells total K18 protein (proteolysed and full length) found in networks was reduced 10- to 20-fold compared with that in uninfected cells. Thus, Ad proteolysis of the K18 head domain or inhibition of host protein synthesis results in reduction of total K18 protein in polymerized networks in infected cells. Most surprisingly, infected cells treated with 2AP contained normal levels of uncleaved K18 protein and only detectable amounts of the 41-kDa cleavage product. The normal level of intact K18 polypeptides present in the insoluble intermediate filament fraction is indicative of its ongoing synthesis and rapid incorporation into structural keratin filaments.

A similar analysis was performed for keratins K1, 5 to 8, 10, 11, and 18, using an antibody that recognizes all species equally

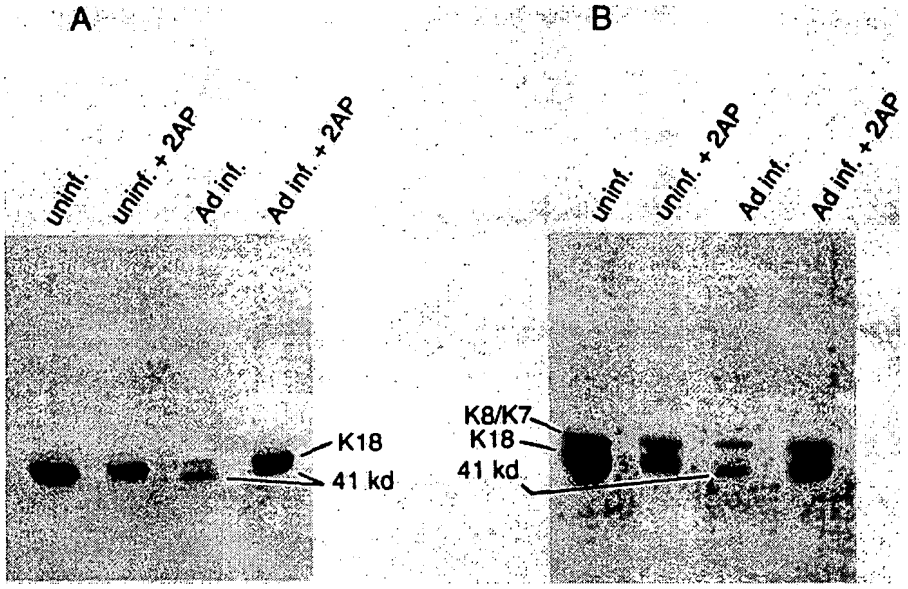

FIG. 7. Western immunoblot analysis of keratin cleavage during late Ad infection in cells treated with 2AP. 293 cells infected with 50 PFU of wt Ad per cell in the absence or presence of 2AP treatment were harvested at 24 h p.i. Whole-cell lysates were prepared, and the insoluble keratin network was purified, resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed by immunoblot analysis with an antibody specific for keratin K18 and the 41-kDa degradation product (A) or an antibody that recognizes keratins K1, 5 to 8, 10, 11, and 18 (B). Specific cytokeratin polypeptides were identified by molecular size, using protein markers as indicated. The high molecular size band present in Ad-infected samples has not been identified, but might correspond to a cleavage product of a larger keratin protein.

well (Fig. 7B). The low-abundance K7 keratin is either not present in 293 cells or could not be separated from K8, which migrates in almost the same position. Keratins K1, 5, 6, 10, and 11 are not found in most cultured or transformed cells, so their absence from 293 cells is not surprising in this case (38). However, it was readily apparent that in addition to proteolysis of K18, the level of K7/K8 was also significantly reduced. It was previously shown that inhibition of cell protein synthesis with cyclohexamide did not significantly alter the pattern of keratin filaments, although the abundance of keratin proteins was not examined (37). Thus, since K8 is not a substrate for the Ad proteinase, these results suggest that viral shutoff of host protein synthesis coupled to cleavage of K18 likely reduces the abundance of a variety of polymerized keratin proteins.

Disruption of the cytokeratin network facilitates release of newly synthesized infectious virus particles. Previous studies showed that 2AP prevented Ad-mediated inhibition of host translation with only a marginal reduction in the kinetics of Ad replication and the yield of infectious virus particles (32), which at first seems to be at odds with the absence of cell lysis observed here. However, measurement of infectious virus yields involved release of particles by mechanical disruption of cells, which is typically performed to maximize recovery of mature virus regardless of whether lysis has occurred. The amount of cell-free virus released into medium in the absence of mechanical cell disruption was therefore investigated. 293 cells were infected with 50 PFU of wt Ad per cell in the presence or absence of 2AP treatment. At 3 to 4 days p.i., approximately 24 h after the majority of cells had lifted, the amount of infectious virus released into the medium was assayed before and after mechanical lysis of cells. Infection of cells and treatment with 2AP could not be extended past 3 to 4 days, because the drug itself began to cause toxicity and cell death in uninfected cells. In the absence of mechanical lysis, approximately 250-fold lower levels of infectious virus particles were released from cells treated with 2AP (Table 1). The majority of infected cells not treated with 2AP lysed within 2 to 3 days of infection, releasing large amounts of newly synthesized infectious virus particles. Thus, cell lysis and release of mature virus particles is facilitated by degradation of the cytokeratin network coupled to translation shutoff and an inability to synthesize new keratin proteins.

We next tested whether coupled shutoff of host protein synthesis and cleavage of cytokeratins truly correlates with development of CPE. Mutant Ad2 *ts*1 (temperature sensitive in the L3 proteinase) was exploited to uncouple the two effects, because it cannot cleave cytokeratins K7 and K18 at the restrictive temperature (39.5°C) but efficiently inhibits host cell protein synthesis. Thus, if the model is correct, 293 cells infected with Ad2 *ts*1 should demonstrate only partial CPE and degeneration of the cytoskeleton at the restrictive temperature for L3 proteinase activity, resulting mainly from translation shutoff.

Cells were infected with Ad2 *ts*1 at permissive (32°C) and restrictive (39.5°C) temperatures. Infection with wt Ad at

TABLE 1. Effect of CPE on release of mature Ad virus particles[a]

Treatment	Yield of wt Ad (PFU/ml)[b]	
	−2AP	+2AP
Mechanical cell lysis	1×10^9	5×10^8
No mechanical cell lysis	4×10^8	2×10^6

[a] Infected 293 cells were harvested at 3 to 4 days p.i. when most cells treated with 2AP had lifted from the monolayer, and the amount of infectious virus released into the medium was determined. Cells were either cleared from the medium by centrifugation (no mechanical disruption) or first lysed by sonication before clarification.

[b] Virus yields in medium were determined by plaque assay on 293 cells and represent the average of several independent experiments.

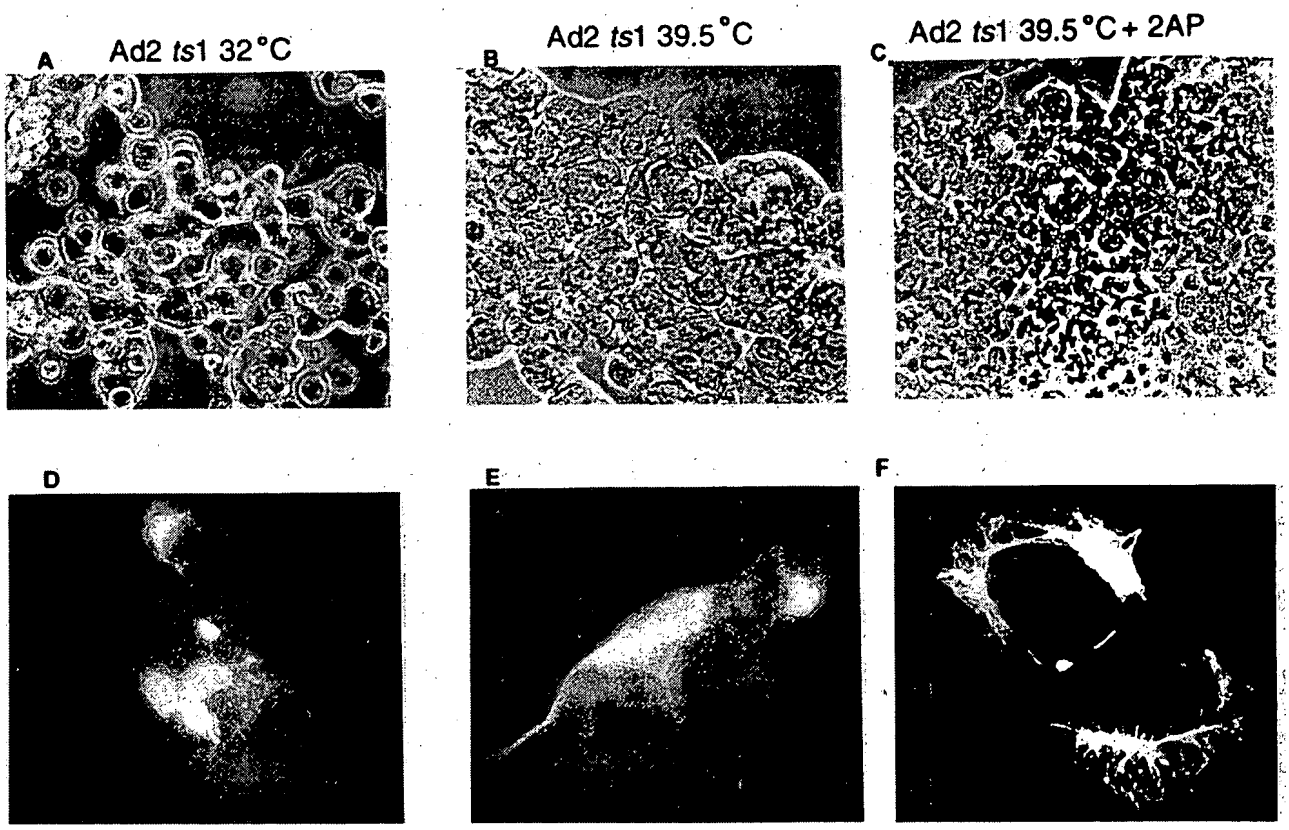

FIG. 8. Disruption of the cytokeratin network requires coupled shutoff of host translation and cleavage of keratin K18. 293 cells were infected with Ad2 *ts*1 at 4,000 particles per cell, a 25 PFU per cell equivalent, given the high particle/PFU ratio for this mutant (10), in the presence or absence of 2AP at 32 or 39.5°C. Cells infected with wt Ad at 39.5°C displayed identical viral growth kinetics as those infected at 37°C (data not shown). Cells were fixed and processed for indirect immunofluorescence, using a K18-specific antibody. Cells infected at 32°C were fixed at 40 h p.i.; cells infected at 39.5°C were fixed at 22 h p.i.

39.5°C showed a growth pattern identical to that of virus at 37°C, and therefore those data were not presented. At the permissive temperature (32°C) the Ad replication cycle is somewhat delayed, and late phase is not fully developed until approximately 36 h p.i. compared with that at 24 h at 37°C. Nevertheless, at 32°C, infection with Ad2 *ts*1 was indistinguishable from that of wt Ad (Fig. 8), causing gross morphological alterations and degeneration of the cytokeratin network as observed earlier. At the restrictive temperature, however, there was a striking difference from wt Ad-infected cells (compare Fig. 6 with 8). Cells infected with Ad2 *ts*1 possessed only partial manifestations of CPE, including slight swelling and rounding (Fig. 8B and E). Interestingly, staining of keratin filaments revealed that the cables were largely intact but poorly resolved. Thus, slight degeneration of the intermediate filament network was evident in the absence of keratin cleavage, similar to that of 2AP-treated cells infected with wt Ad. In cells infected with Ad2 *ts*1 at 39.5°C and treated with 2AP, the keratin network was remarkably well preserved (Fig. 8C and F), resembling that of uninfected cells rather than that of wt Ad-infected cells treated with 2AP. Distinct keratin cables were clearly visible despite productive Ad infection, and cells appeared almost identical to uninfected controls. Accordingly, at 39.5°C, the Ad2 *ts*1-infected cells only detached from the monolayer quite late in the infection and lysed more poorly than cells infected at the permissive temperature for L3 proteinase activity (data not shown). Precise quantitation of the number of Ad2 *ts*1 particles released from cells under these conditions was not possible, however, given the impaired ability of the virus to form plaques even at the nonrestrictive temperature (10; unpublished results).

DISCUSSION

A primary function for Ad inhibition of host translation was elucidated in this report. Through several lines of evidence it was shown in tissue culture cells that shutoff of host protein synthesis was only slightly (severalfold) involved in selective translation of late viral mRNAs and instead was necessary to facilitate cell lysis and release of infectious progeny virus particles. First, there was a striking absence of CPE in late Ad-infected cells if shutoff of host translation was prevented by 2AP (Fig. 2 and 4) or in cells which are resistant to viral translation inhibition (Fig. 1). Since only slightly reduced levels of virus particles were synthesized (~two- to fourfold), it was apparent that efficient translation and accumulation of late viral polypeptides did not require shutoff of host protein synthesis and that accumulation of late polypeptides was not necessarily cytotoxic. Second, the absence of CPE was associated with an inability to efficiently release virus particles from infected cells (Table 1). The Ad particles retained in cells were fully matured and infectious, as evidenced by the normal yields

obtained if cells were mechanically lysed. Third, the Ad L3 23-kDa proteinase was shown previously to degrade K18 and probably K7 keratin proteins late during Ad infection, leading to loss of cell structural integrity (10). In Fig. 6 and 8 we showed that degeneration of the cytokeratin network, appearance of CPE, and loss of cell structural integrity only occurred if Ad prevented host protein synthesis. Biochemically, Ad pathogenic effects correlated with proteolysis of keratin K18 and with an inability of the cell to maintain the integrity of the cytokeratin network through continued synthesis of new proteins (Fig. 7 and 8). It is important to note that synthesis of keratin K18 alone is probably insufficient to prevent CPE and cell lysis and that other host polypeptides may also be required. Additionally, although cleavage of keratin K18 coupled to translation inhibition is clearly important for CPE, it is unlikely to be solely responsible for the Ad-induced effect.

2AP was shown to prevent Ad shutoff of host protein synthesis by preventing virus-mediated dephosphorylation and inactivation of translation factor eIF-4E, also known as cap binding protein (32, 33). Unlike most cell mRNAs, late Ad mRNAs appear to require only minute amounts of this factor (16, 51). However, the basis for 2AP activity in translation shutoff is not known, and several other interpretations of our results should therefore be considered. First, we can currently exclude the possibility that the activity of 2AP was unrelated to its effect on Ad translation shutoff. For instance, it is clear that the L3 23-kDa proteinase was not simply inactivated by 2AP treatment. This proteinase is essential for morphogenesis of viral structural proteins and assembly of virus particles (7, 64), which were produced at near-normal levels despite 2AP treatment (Fig. 2 and Table 1). Thus, the L3 23-kDa proteinase retained its activity in the presence of 2AP, but large amounts of degraded keratin filaments did not accumulate. Second, it is clear that 2AP did not act to enhance the synthesis of new keratin proteins in uninfected cells (Fig. 7). Third, it is unlikely that 2AP treatment resulted in modification of keratin K18 in such a way that it was resistant to cleavage by the Ad proteinase, because the same recognition site is present in several late Ad structural proteins which were efficiently cleaved in the presence of 2AP. Finally, further evidence that 2AP prevents cytokeratin degradation by blocking late Ad translation shutoff derives from its inability to prevent shutoff of host protein synthesis when added more than 2 h p.i. (32). The later addition of 2AP failed to block both development of CPE during Ad infection and proteolysis of the cytokeratin network (data not shown). Additionally, 2AP prevented even the milder form of CPE apparent in cells infected with Ad2 *ts*1, which inhibits host translation without cleavage of keratin cables. Thus, it is doubtful that 2AP possesses an intrinsic ability to prevent Ad-mediated degradation of cytokeratin filaments apart from its ability to block translation shutoff by the virus.

The cytokeratin network is generally considered to be quite stable, with polymerized keratin proteins such as K18 possessing half-lives on the order of 3 to 4 days (15). Evidence also indicates that there is little free pool of unpolymerized, soluble cytokeratin proteins (reviewed in reference 8). Thus, the stability of the keratin network can be readily impaired by the introduction of only small amounts of an amino-truncated keratin protein (1) or cleavage of the head domain of keratin K18 by the Ad L3 23-kDa proteinase (10). Importantly, inhibition of cell protein synthesis does not cause degeneration of the keratin network (37), excluding Ad translation shutoff per se as the mitigating effect.

In contrast to the image of the cytokeratin network as static and easily disrupted, there is also good evidence that it can be a dynamic structure, disassembling and reassembling with mitosis in some cells (23, 36). Limited evidence from in vitro studies suggests that disassembly may be regulated by specific phosphorylation of certain keratin proteins (9, 63). In vivo, reorganization of the network occurred by epidermal growth factor-induced phosphorylation of a 55-kDa keratin (5), although disassembly induced by phosphorylation has not yet been observed in experimental systems in vivo. Regardless, it is unlikely that 2AP acted by altering cytokeratin phosphorylation. The viral L3 proteinase clearly mediated disassembly of the cytokeratin network and induction of CPE during late Ad infection when coupled to translation shutoff, since the L3 23-kDa mutant (Ad2 *ts*1) failed to degrade cytokeratin filaments at the restrictive temperature (10) (Fig. 8) and caused only mild CPE in 293 cells. In addition, 2AP had no effect in uninfected cells or when added later than 2 h after Ad infection. Our results are most consistent with a model in which inhibition of host translation is required to prevent continued insertion of new keratin proteins into filament networks impaired by Ad proteolysis.

Several studies support a model in which continued synthesis of keratin proteins could prevent collapse of the filament network despite cleavage of K18. The cytokeratins, like most intermediate filaments, have been shown to exist in a dynamic equilibrium in which a small free pool of unpolymerized subunits can rapidly exchange into polymerized filaments (reviewed in reference 47). For example, microinjected keratin K18 was fully incorporated into the cytokeratin network of epithelial cells by 1 to 2 h after introduction (37). The very rapid incorporation of free keratin protein into authentic filaments in epithelial cells was also observed after microinjection of K8 and K18 mRNAs, which showed a clustered pattern of new protein distribution (22, 24). It was suggested that newly synthesized keratins were preferentially or cotranslationally incorporated into existing cytokeratin filaments. Cleavage of keratins K18 and K7 during Ad infection would therefore be expected to only marginally alter the keratin network if synthesis of new keratin proteins was not prevented, since it is likely that those proteolysed would be rapidly replaced. In support of this model, wt Ad-infected cells treated with 2AP were found to possess a slightly disassembled network of cytokeratins (Fig. 6), fewer distinct cables than in Ad2 *ts*1-infected cells, and small amounts of the 41-kDa K18 cleavage product (Fig. 7).

Previous work from our laboratory suggested that Ad shutoff of host protein synthesis involves a different molecular mechanism than that used to promote selective translation of late Ad mRNAs (reviewed in reference 46). Separate control for translation of late Ad mRNAs and inhibition of host protein synthesis can be envisioned to provide flexibility in the viral life cycle. For example, it is well established that Ad can chronically infect lung bronchial lining and intestinal mucosa of humans for periods of months or years, resulting in the continuous shedding of low levels of virus (21, 49; reviewed in reference 48). It is possible that chronicity might be partly mediated by a failure of the virus to inhibit cell translation or to degrade the cytoskeleton. This would be expected to result in slower and poorer lysis of infected cells and a gradual release of smaller numbers of infectious particles. In addition, it is possible that these cells might be more efficiently cleared by cell-mediated immune mechanisms before they ultimately die since viral antigens would be presented on the surfaces of infected cells for considerably longer periods of time. It should be possible to test the role of cell structural integrity in the release of Ad particles and development of chronicity in cotton rats, an animal model for human pulmonary infection by Ad

(29, 41, 43). As shown by Ginsberg and colleagues, the pulmonary temperature of cotton rats is near the restrictive temperature for many Ad *ts* mutants (28). These studies demonstrated that Ad early gene expression was sufficient to induce viral pneumonia but that more extensive pathology developed when Ad was permitted to enter the late phase of its replication cycle. Infection of cotton rats with a mutant Ad deleted of the E1B 55-kDa protein, which is required for expression of Ad late genes and shutoff of cell protein synthesis (4, 42), was reported to produce significantly less pathology (28). These results led to the suggestion that the shutoff of host protein synthesis might be a critical part of virus-induced pneumonia. Future studies should now more directly investigate the influence of Ad translational control and virus-mediated degeneration of the cytoskeleton on the pathogenicity of infection in animal models.

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Exhibit 5

ARTICLES

Study of Adenovirus Production in Serum-Free 293SF Suspension Culture by GFP-Expression Monitoring

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The red-shifted S65T mutant green fluorescent protein (GFP) was used to compare the adenovirus (Ad) production and post-infection survival of 293SF and 293S cells in serum-free and serum-containing flask cultures, respectively. The GFP-expressing vector permitted the quantification of both the level of GFP expressed by infected cells and the infectious viral content of the cultures by flow cytometry in a simple, fast, sensitive, and reliable way. The GFP has the main advantage of fluorescing without any substrate addition. Infected cultures showed the coexistence of two populations of fluorescent cells, high-fluorescence cells (HFCs) and low-fluorescence cells (LFCs), in proportions that varied between 20 and 75 hpi. The gradual increase in the number of LFCs at the expense of HFCs correlated well with the increase in the number of dead cells. This relationship could be used for the continuous measure of a culture's viability with the appropriate on-line instrumentation. The post-infection death rate of infected 293SF cells was higher than that of infected 293S cells, but the level of GFP fluorescence in viable, highly fluorescent cells was similar in the two infected cell lines. The number of infectious viral particles (IVPs) was quantified in less than 24 h by an infection assay of 293S cells in wells with viral particles extracted from the culture samples, and the results were more reproducible (±10% variation) than those generally reported for conventional plaque assay titrations or end-point dilutions. The viable cell-specific IVP concentrations were for most experiments similar, indicating again that the difference between the two cell lines was their unequal post-infection viabilities, not the virus production by the infected living cells.

Introduction

The popularity of recombinant adenovirus vectors (AdV) for gene therapy is constantly increasing, causing a high demand for methods of production of large, homogeneous, and safe lots of vectors. The development of such technologies involves the culture of 293 (human embryonic kidney) cells, a cell line well-known for its ability to support the replication of the E1A+E1B-defective adenoviral particle, at the highest cell density possible without loss of product yield, in a large process bioreactor unit, and in serum-free medium (SFM). One important impediment to the large-scale production of viruses, in addition to the fact that AdV is harvested with intact cells, is the maintenance of the post-infection cell survival during virus amplification leading to maximum AdV titer. The anchorage-dependent 293 cells growing on microcarriers detach easily when infected with viruses such as AdV, which impedes the process scale-up. Conversely, shear effects are less a concern when cells are adapted to suspension and cultured in conventional large stirred-tank bioreactors. Therefore, the development of the 293N3S cell line, adapted to suspension culture, was an important achievement (Graham, 1987), even with the cell line's doubling time of 30 h. Recently, useful results were obtained in our laboratories with suspension-adapted 293S cells (Garnier et al., 1994; Nadeau et al., 1996). Compared to 293N3S cells, 293S cells have a lower tendency to form aggregates and grow to a higher density in suspension. In batch culture, 293S cells can grow to up to 5×10^6 cells/mL with a doubling time of 20 h during the exponential growing phase, and an efficient virus production can take place at 2×10^6 cells/mL in fed-batch culture (Garnier et al., 1994).

A central issue in the development of a human-injectable product is the elimination of ill-defined and potentially harmful components, such as serum, from the culture medium. Peshwa et al. (1993) developed the first SFM for modified 293 cells. They reported that the cells spontaneously adapted to the SFM and rapidly reached densities of 3.5×10^6 cells/mL in batch cultures of aggregates. Berg et al. (1993) adapted 293 to suspension culture with loosely associated aggregates with a generation time of 36–40 h and the cells reached a maximum viable cell density of 2.5×10^6 cells/mL. However, we did not succeed in easily growing 293S cells in SFM. A long procedure of weaning and two steps of screening were required to obtain a clonal and stable 293S line that could grow in SFM with little clumping and could support AdV infection (Côté et al., submitted for publication). This SFM-adapted cell line, named 293SF, can grow to up to

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8×10^6 cells/mL in batch and support an efficient virus production at up to 1×10^6 cells/mL. Although 293SF cells grow well in suspension, the infected-cell viability decreases faster without serum (50% viability after 48 h post-infection (hpi)) than in serum-containing medium. However, serum effect on intracellular viral replication or virus attachment to the cell membrane during infection process is not documented in the prevailing literature.

The AdV titer can be estimated by different techniques, such as plaque assays or end-point dilution assays, but these methods are long, tedious, and imprecise (Mittereder et al., 1996). The AdV titer represents only a small fraction of all the AdV produced in a culture. Most of the viral particles appear to be noninfectious. The reported bioactivity of a viral stock may vary from as little as 1% to up to 50%, depending on the method and conditions of the quantification (Mittereder et al., 1996; Shabram, 1996). Since a vector destined for gene therapy must be highly infectious, it is necessary to develop a production process that takes this limited bioactivity into account. Infectious viral particles (IVPs) are those which can invade cells and eventually constrain them to express a gene of interest. Merely increasing the total number of viral particles produced in a large-scale culture is not enough; the bioactivity of the viral stock must also be kept as high as possible during the whole production process, including the downstream processing. The total virus concentration can be estimated by HPLC, but the overall virus bioactivity, defined as the percentage of IVPs in a population of viral particles, is an important parameter. This is particularly true for downstream processing leading to pure and infectious viral particles needed for clinical trials of gene therapy. A tool is necessary to achieve this goal and facilitate the detection of a single infection event during the AdV titration of multiple samples generated during the bioprocess development.

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has become an important marker of gene expression (Chalfie et al., 1994). The GFP has also been identified as a potentially powerful fusion tag for protein localization and translocation studies in intact cells (Yokoe et al., 1996). Although the expression and detection of the wild-type GFP was reported to fail in mammalian cells (Steams 1995), the expression of brighter proteins by variants is now possible (Cheng et al., 1996; Zolotukhin et al., 1996; Crameri et al., 1996). In this study, we used the red-shifted S65T mutant GFP, which has a fluorescent intensity approximately 6 times higher than that of the wild-type GFP (Heim et al., 1995). The mutant protein allows the detection of fluorescence as early as 6 hpi. Contrary to the β-galactosidase gene, which is frequently used as reporter gene, the GFP does not require substrate addition to be detectable, is powerful enough to reveal real-time events in individual cells, by either fluorescent microscopy or flow cytometry (FCM), and remains intracellular for viable infected cells. The use of FCM allows the monitoring of GFP expression over time on a single-cell basis, which can be a precious tool for the study of virus production.

In this paper, we compare the production capabilities of 293SF and 293S infected with the Ad-GFP vector. Both GFP and virus production were monitored using FCM. The time course of GFP production revealed the relationship between loss of cell viability during the infection phase and protein expression. The fluorescent vector permitted us to estimate the viral titer in a simple, fast, sensitive, and reliable way. Typically, titer estimations required only overnight incubation instead of the 10−14 days for plaque assays.

Materials and Methods

Cell Culture. The 293S cells were kindly provided by Dr. Michael Matthew (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY). The cells were maintained in suspension culture in a custom-made calcium-free Dulbecco's Modified Eagle medium (CFDMEM; American Bioorganics Inc., Niagara Falls, NY) supplemented with 5% (v/v) COSMIC bovine calf serum (BCS; Hyclone, Logan, UT) and 0.1% (w/v) Pluronic F68 (GIBCO, Grand Island, NY) as described previously (Garnier et al., 1994; Nadeau et al., 1996). Procedures for cell maintenance in shake flasks and counting are described by Nadeau et al. (1996). The 293SF cells were derived from 293S cells. They were adapted to low-calcium serum-free medium (LC-SFM) which is a custom-made low-calcium H-SFM (GIBCO, Grand Island, NY) as described by Côté et al. (submitted for publication). LC-SFM was supplemented with 0.1% (w/v) bovine serum albumin (BSA #A7030; Sigma, St. Louis, MO) and 1% (v/v) chemically defined lipids (GIBCO) to get the complete LC-SFM. 293S cells typically grew to up to 5×10^6 cells/mL, and 293SF cells reached 8×10^6 cells/mL, in 50-mL shake flasks containing 12 mL of culture. The AROS 160 orbital shaker (Barnstead/Thermolyne Corp., QC, Canada) was operated at 100 rpm and had an orbit radius of 3.2 cm.

Ad-GFP. The recombinant adenovirus was constructed as described by Couture et al. (submitted for publication). Briefly, the construction was similar to the previously described Ad5 CMV-LacZ (Acsadi et al., 1994) but with some modifications, including the replacement of the LacZ gene by the S65T GFP mutant gene (Heim et al., 1995). This variant produced the S65T mutant GFP which had long wavelengths of excitation and emission (490 and 510 nm) as well as an increased emission signal intensity detectable in single cells, by either fluorescent microscopy or FCM.

Infection and Sampling. The infection experiments were performed in 125-mL polycarbonate shake flasks (CORNING, NY) at working volumes of 35 mL. The cells were maintained in exponential growth phase and then centrifuged and resuspended in fresh medium prior to infection. Infections were done at 1×10^6 cells/mL with a multiplicity of infection (MOI) of 10 IVPs per cell. Static controls were prepared by transferring infected cells from some shake flasks to 25 cm^2 T-flasks. The infected cultures were incubated at 37 °C in an atmosphere of 5% CO_2 (Forma Scientific, Marietta, OHIO). Aseptic samples of 1.3 mL were taken twice daily; 300 μL was used for haemacytometer cell counts with erythrosin B exclusion; 500 μL of the cell suspension was transferred in 15-mL sterile tubes; 500 μL was placed in Eppendorf tubes and centrifuged at 500*g* for 2 min (Savant Instruments Inc., Hicksville, NY). The total-suspension samples and centrifugation supernatants were kept at −80 °C for further AdV titration. The cell pellets were prepared for FCM analysis. Cells were resuspended by gentle manual shaking, and 500 μL of cold phosphate-buffer saline (PBS) was added. The cells were then fixed for FCM as described below.

Analysis by FCM. PBS (500 μL) containing 4% paraformaldehyde was added to the cell suspension drop by drop under constant gentle agitation to prevent cell aggregation; the cells were incubated at 4 °C for 30 min, centrifuged, and washed once with cold PBS; the cells were then resuspended in 500 μL of cold PBS and assayed

by FCM. We assayed the same samples immediately after fixation and once a day until the third day. The fluorescence intensity decreased by 30% during the first 12 h and then remained stable for at least 3 days at 4 °C while the fraction of HFCs decreased by less than 7%, indicating that GFP content remains relatively constant during storage at 4 °C. GFP expression during cell infection is at maximum for freshly fixed samples while the distribution of high-fluorescence cells (HFCs) and low-fluorescence cells (LFCs) is quite stable over a 3-day incubation period. We found that accumulating fixed-cell samples at 4 °C and assaying them all at the same time gave the most accurate results.

Suspensions of paraformaldehyde fixed cells were analyzed by FCM using a Coulter Epics Profile II (Coulter Electronics, Hieleah, FL) equipped with a 15-mW argon-ion laser emitting at 488 nm, as described by Mosser et al. (1997). Ten thousand events were analyzed for each sample. Debris and aggregates were excluded from the analysis on the basis of the forward-angle light scatter (FS) and the side-angle light scatter (SS). Only single cells were analyzed. Green fluorescence of the S65T mutant GFP was detected using a 550-nm long-pass dichroic and a 525-nm band-pass filter. Light scatter values were measured on a linear scale of 1024 channels and fluorescence intensities on a logarithmic scale of four decades.

Positive regions of analyses were set on the histogram considering infected cells at 4 hpi as the negative control. The 293SF cell sample at 48 hpi was used to determine boundaries of well-defined LFC and HFC populations. Results are reported as the mean channel of fluorescence and as a percentage of positive cells. The day-to-day consistency of measurements was checked by Standard-brite microspheres (Coulter Electronics, Hialeah, FL).

The fluorescence intensity was an indicator of the GFP level produced over the infection period (GFP-positive cells). The GFP signal distribution which extends over more than one log range represents the variation of the number of IVPs per cell, or MOI, as well as the variation in time since the infection effectively occurred following AdV-cell contact.

Ad-GFP Titer by Infection Assay of 293S Cells in Wells. A biological method was used to measure the number of mature virions (IVPs) in a sample by taking into account virus penetration as well as viral gene expression. A similar approach was correlated to standard plaque assay by Couture et al. (submitted for publication). In our study, each well of a 12-well plate was seeded with 1.0×10^6 293S cells. To each well was added 2 mL of CFDMEM containing 5% BCS. The frozen total-suspension samples of the shake flask and T-flask cultures were submitted to three freeze–thaw cycles to release the Ad-GFP from the cells. The frozen culture supernatants were also thawed (once) and then diluted in fresh serum-containing medium. To each well was added 5, 10, 25, and 50 μL of diluted samples. After an incubation at 37 °C and 7% CO_2, the cells were harvested (1 mL from each well) at 16–18 hpi, fixed with paraformaldehyde as described above, and analyzed by FCM immediately. This short infection period allowed the virus to enter the cells and express the GFP at a level detectable by FCM. At 18 hpi there is no viral amplification (mature virions), but viral genes are expressed and can be detected. Some samples were observed under the fluorescence microscope. Fluorescent cells (FCs) were visible this way, but at least twice as many could be detected in the same samples by the more sensitive FCM (data not shown).

The percentage of FCs increased linearly with the amount of virus added in wells in which no more than 50% of all cells were infected. Each FC received at least one IVP, and if a Poisson distribution is assumed, most of all the FCs contained only one IVP when less than 50% of the total cell population was infected. The viral titers are therefore reported only for wells containing no more than 50% FCs. They were calculated the following way:

$$\text{Ad-GFP titer (IVPs/mL)} = \frac{\%\text{ of FCs in well}\cdot\text{dilution factor}\cdot X}{100\cdot\text{sample volume per well}} \quad (1)$$

$$\text{specific viral production (IVPs/cell)} = \frac{\text{Ad-GFP titer}}{\text{sample total cell density}} \quad (2)$$

$$\text{HFC-specific viral production (IVPs/HFCs)} = \frac{\text{specific viral production}}{\text{HFC fraction}} \quad (3)$$

where X is the number of cells per well. For this infection assay, we considered the percentage of fluorescent cells only, not the intensity of fluorescence in fluorescent cells, since only one IVP was assumed to enter into a single cell in most instances.

Results and Discussion

GFP Expression by Cells after Infection. Figure 1 shows flow-cytometric profiles of 293 cells during infection. The negative controls (Figure 1a,a') correspond to 293S and 293SF cells that have just been infected by Ad-GFP (less than 4 hpi) respectively. The HFC peak on Figure 1b was observed with 293S cells at 24 hpi. This intensity of signal is close to the maximum observed under these conditions for both cell lines (Figure 1b for 293S cells and Figure 1b' for 293SF cells). Figure 1c shows a population profile of 293S cells at 48 hpi. Figure 1c' represents the population profile of 293SF at 48 hpi, when the cell viability was around 50%. Two peaks appear, one HFC peak equivalent to that of Figure 1b,b' and a LFC peak of cells of inferior GFP content and of mean intensity almost 2 orders of magnitude lower than that of the HFC peak. Samples taken at other times show correspondingly intermediate ratios of HFCs to LFCs. In all samples collected after 20 hpi, a linear correlation ($r^2 = 0.95$) was observed between the proportion of HFCs and the fraction of viable cells determined by haemacytometer counts (Figure 2). Figure 2 also shows an inverse linear relationship ($r^2 = 0.94$) between the proportion of LFCs and the fraction of viable cells. Therefore, the LFC peak observed following the maximum GFP expression around 24 hpi is proportional to the population of dead cells, whereas the HFC peak represents the fraction of viable cells. Presumably, a fraction of the GFP content of the viable infected cells leaked at death, resulting in dead cells of lower fluorescence. However, the dead-cell fluorescence signal did not reach the values of the negative control. The mean signal decreased faster in 293SF cells than in 293S cells. The correspondence between the fraction of HFCs and the percentage of viable cells could be used as a tool to determine the viability of an Ad-GFP infected culture. With the appropriate equipment, this could be done on-line, which is a net advantage over haemacytometer counts.

Post-infection Cell Survival and Fluorescence. Figure 3a shows the changes in the fraction of HFCs after infection in shake flask and T-flask cultures, whereas the

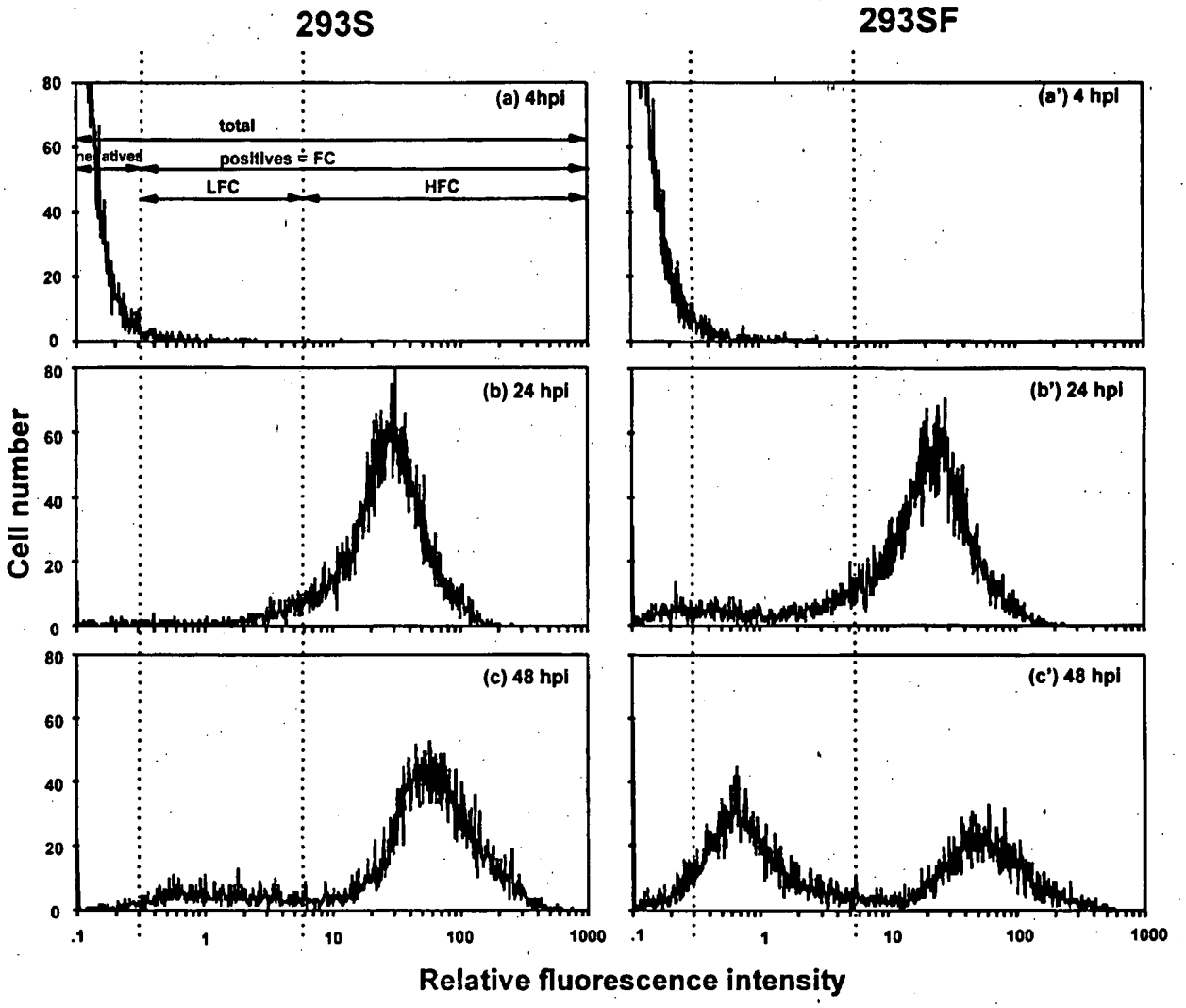

Figure 1. Flow-cytometric analysis of 293S and 293SF cells, respectively, infected with Ad-GFP at an MOI of 10 IVPs per cell at (a) and (a') 4 hpi (negative controls); (b) and (b') 24 hpi; (c) 48 hpi when 293S cell viability was 84% and (c') 48 hpi when 293SF cell viability was around 50%.

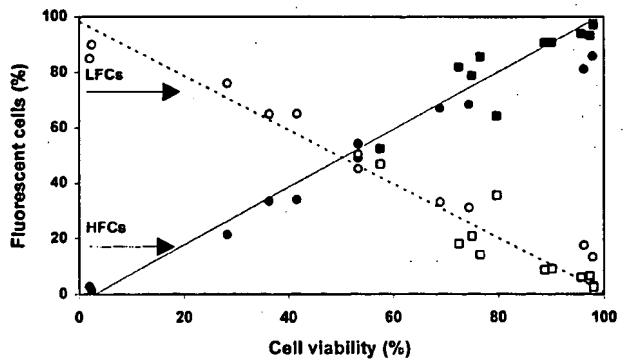

Figure 2. Fraction of fluorescent cells immediately after paraformaldehyde treatment as a function of cell viability of an independent shake flask experiment. 293S cells (squares) and 293SF cells (circles). Open symbols: low-fluorescence cells; closed symbols: high-fluorescence cells. Samples were collected after 20 hpi to allow the expression of GFP in infected cells.

changes in the fraction of LFCs are shown on Figure 3b. Between 24 and 75 hpi, the two quantities may be interpreted as the fractions of viable cells and dead cells, respectively. (Values before 4 hpi are not representative of cell state, since the GFP was not produced or detectable at that time.) The death rate of 293S cells was substantially lower than that of 293SF cells over the 3-day infected period. The viability of 99% at infection decreased after 3 days to about 70% for 293S cells and 15% for 293SF cells. Cell viability profiles were similar for infected cells in static and agitated cultures. This indicates that cell death was more related to the infection itself than to any stress caused by agitation, especially in serum-free conditions.

Figure 4a shows that the mean fluorescence of all fluorescent 293S cells increased rapidly in the first 24 hpi, increased more slowly between 24 and 48 hpi, and decreased afterward, whereas the mean fluorescence of all fluorescent 293SF cells peaked at 24 hpi, after which it decreased. In both cultures, the decline in mean fluorescence was owed to the gradual increase in number of low-fluorescence dead cells. When only the mean fluorescence of infected viable cells is considered (Figure 4b), it can be seen that similar amounts of GFP were produced at similar rates in all conditions by both cell lines, although 293SF cells were on average slightly less productive in HFCs.

Production of IVPs. The infection assay in 12-well plates done by addition of four different volumes per sample showed titer standard deviations of less than 10% for wells where no more than 50% of cells were fluorescing. Those positive cells were counted by FCM when detected in the FC region (Figure 1a). The highest final

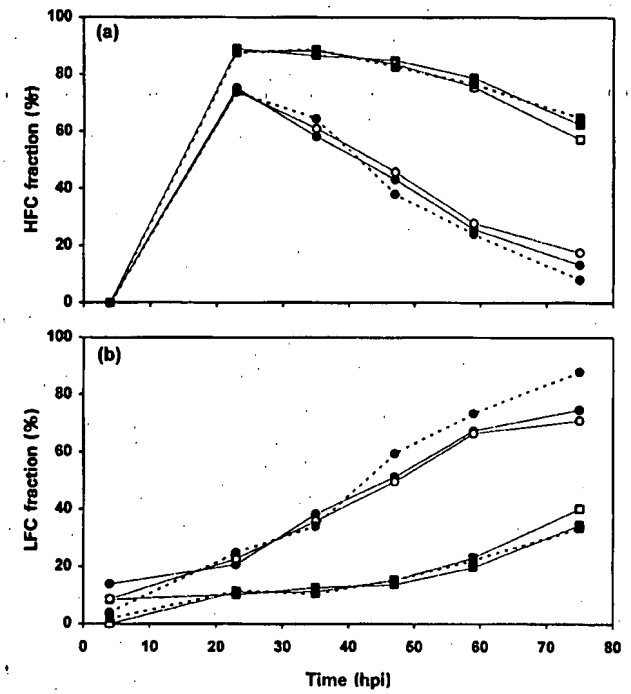

Figure 3. Time course of (a) high-fluorescence-cell fraction and (b) low-fluorescence-cell fraction after infection in all experiments. 293S cells (squares) and 293SF cells (circles) in T-flasks (dashed lines) and shake flasks (solid lines). Open and closed symbols are duplicates.

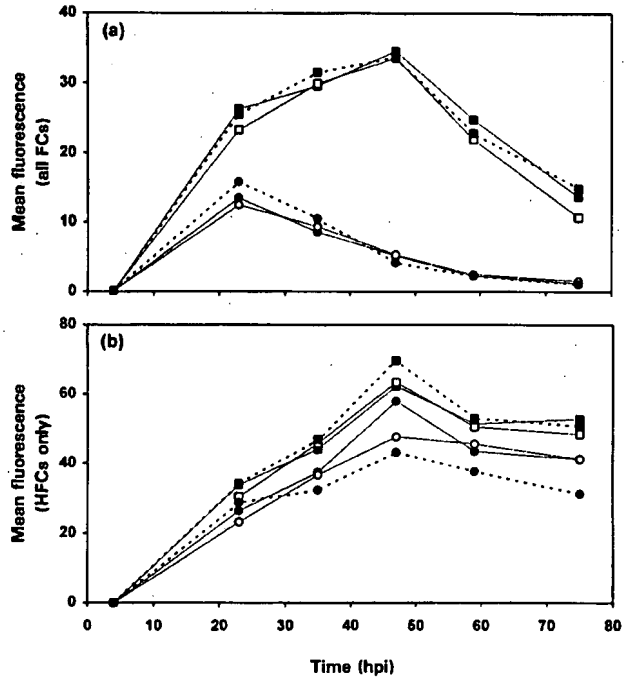

Figure 4. Mean fluorescence of (a) all fluorescent cells and (b) high-fluorescence cells. 293S cells (squares) and 293SF cells (circles) in T-flasks (dashed lines) and shake flasks (solid lines). Open and closed symbols are duplicates.

Ad-GFP titer with the method used in this study, 350 IVPs/(10^6 total cells), was obtained with total-static 293S cells in the presence of serum in T-flasks (Figure 5a). In the shake flask samples, 220 IVPs/(10^6 total cells) were measured with 293S cells in serum-containing medium, whereas 293SF cells in SFM yielded about 100 IVPs/(10^6 total cells). But the HFC-specific IVP concentrations (Figure 5b) were for most experiments similar until 48 hpi, indicating here also that the difference between the

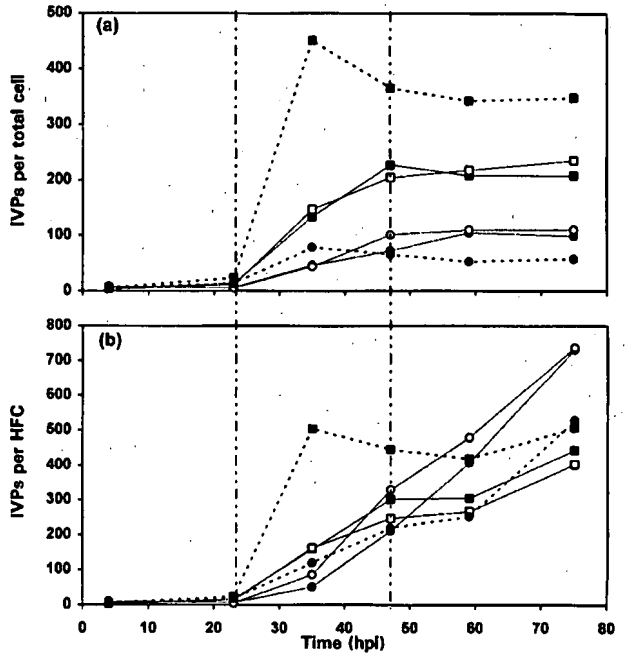

Figure 5. Specific total-suspension infectious viral particles (IVPs) measured by assay infections in wells. 293S cells (squares) and 293SF cells (circles) in T-flasks (dashed lines) and shake flasks (solid lines). Open and closed symbols are duplicates.

two cell lines was their unequal viabilities. The virus productions in the infected viable cells were similar in all cases except with 293S in T-flask, suggesting that AdV production may be influenced by the agitation stress even if the cell viability remains high. In our experiments, as long as they were alive, infected 293SF cells produced infectious virions as well as 293S cells in suspension culture. No significant difference was noticed between static and suspension culture of infected 293SF cells. Since the Ad-GFP titer at 24 hpi was still about equal to the MOI at infection (10 IVPs per cell), the subsequently produced IVPs came from cells that were alive after 24 hpi; the dead cells did not produce infectious viral particles. After 48 hpi, the apparent increase in the HFC-specific Ad-GFP concentration owed to the gradual decrease in the number of viable cells as they died since the total population-specific titer remained constant during that time (Figure 5a).

The Ad-GFP vector described here should be very useful in the development of a process for the production of adenoviral vectors of similar construction. However, other vector constructions may result in different patterns of replication and expression within the cells. It may be possible to express GFP in fusion with a protein of the viral coat without altering the infectivity of the particles. The resulting fluorescent viral particles might then be detectable with a sufficiently sensitive probe. The GFP signal would then be independent of the recombinant protein of interest expressed by a given vector. Also, on-line monitoring of GFP expression may be useful for studying the kinetics of adenoviral production by infected suspension cultures. In fact, coupling the signal to a mathematical model that takes into account the delay between GFP expression, the appearance of fluorescence, and AdV production may allow a more precise determination of the harvesting time, resulting in an improved yield of active product.

Conclusion

GFP monitoring by FCM is a useful method for the analysis of the virus production in culture that avoids

the multiple steps usually needed to label a product of interest. The fact that a reproducible virus quantification can be quickly done is of value for process study.

The level of GFP in viable highly fluorescent cells was similar in serum-containing and serum-free cultures of 293S and 293SF cells, respectively, infected with the adenovirus. The culture mean fluorescence decreased in a manner that correlated well with the appearance of a population of low-fluorescence dead cells. These findings could be used for the continuous assessment of a culture's viability with the appropriate on-line instrumentation.

Viable infected 293SF cells produced the same amount of recombinant protein and infectious recombinant adenovirus as viable infected 293S cells in suspension culture, but the post-infection survival of 293SF cells was shorter. We are presently conducting studies to improve the cell viability following infection in serum-free conditions.

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